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Recombinant Complement Factor H in Complement-Mediated Diseases

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Abstract

Complement Factor H (FH) is a 155-kDa plasma glycoprotein that regulates the alternative pathway of the complement system. Mutations or deficiencies in FH are associated with some cases of the kidney diseases C3 glomerulopathy and atypical haemolytic uraemic syndrome (aHUS). Recombinant human FH could potentially be used in some patients as a therapy for these diseases, both of which confer significant morbidity. This requires animal trials as well as a better understanding of which patients are likely to benefit most from FH-supplementation therapy.

An abundance of FH in the plasma of healthy individuals implies that high doses of any potential recombinant FH protein will be needed. Previously, *Pichia pastoris* containing codon-optimised DNA encoding human FH yielded useful but relatively small quantities of protein. In other experiments plasma-derived murine and human FH rapidly controlled C3 turnover in the murine model of C3 glomerulopathy, a FH-deficient mouse strain. Difficulties in obtaining large quantities of pure well-characterised murine FH and the rapid development of an immune response to human FH prevented longer-term trials of these agents.

This study aimed to generate recombinant murine FH (rmFH) in sufficient quantities for thorough functional and biophysical characterisation and subsequently *in vivo* studies in FH-deficient mice. It further aimed to enhance production of recombinant human FH to allow assessment of the functional implications of disease-linked mutations.

The methodology that had yielded recombinant human FH in *P. pastoris* generated only minimal quantities of rmFH. Bearing in mind the 40 disulfides present in FH, a new host *P. pastoris* strain was developed containing an expression-optimised gene for protein disulphide isomerase (PDI). This produced multi-milligrams of rmFH per litre. The rmFH had an affinity for both human and murine C3b similar to native mFH and was functional in co-factor and decay acceleration complement assays.

The rmFH was also functional in the FH-deficient mouse model – it increased plasma C3 levels and reduced glomerular C3 staining 24 hours after a single intra-peritoneal injection. The half-life of circulating rmFH was, however, short, and the mice developed antibodies against the recombinant protein after ten days of daily dosing. Despite this, a reduction in glomerular C3 was still seen at ten days implying rmFH may have persisted for longer in the glomerular basement membrane.

Mutations in FH are implicated in aHUS, a life-threatening kidney disease. Recombinant human FH was made with variations in both the N (I62V, R53H) and C terminus (D1119G, S1191L V1197A), and tested in assays for decay-acceleration, co-factor and complement-mediated haemolysis of (self-like) sheep erythrocytes. The addition of PspCN, a bacterial binding protein, enhanced co-factor activity and haemolysis protection, and therefore may reverse the deficiencies of some FH mutant variants.

Lay summary

The complement system is critical for clearance from the body of dying or damaged human cells that can otherwise trigger an inflammatory response leading to disease. Factor H is an important regulatory protein in the complement system. In some people mutations in the FH gene or deficiencies in the protein cause serious kidney diseases, C3 glomerulopathy and atypical haemolytic uraemic syndrome. In other individuals, these diseases are linked to mutations in alternative complement proteins.

It may therefore be possible to treat selected patients with these diseases by boosting their levels of fully functional FH protein. I have discovered a method for biomanufacturing large quantities of both the mouse and human versions of the FH protein in yeast. The mouse version reduced disease symptoms in an animal model of C3 glomerulopathy. Several mutants of the human FH versions were likewise produced and tested to help define which patient cohorts are likely to benefit most from administration of FH.

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Declaration

Unless stated in the text, the work described in this thesis is my own work and has not been submitted for in whole or in part for a degree or other qualification at this or any other university.

Heather Kerr

Abbreviations

ABC	ammonium bicarbonate
ACN	acetonitrile
AFU	Arbitrary Fluorescence Unit
aHUS	atypical haemolytic uraemic syndrome
AMD	age-related macular degeneration
AOX	alcohol oxidase
AP	alternative pathway
BMG	buffered minimal glycerol
BMGY	buffered glycerol-complex medium
BMMY	buffered methanol-complex
bp	base pair
BSA	bovine serum albumin
C3GN	C3 glomerulonephritis
C4BP	C4 binding protein
CCP	complement control protein
cDNA	complementary deoxyribonucleic acid
CFH ^{-/-}	homozygous factor-H deficient mice
CHA	ceramic hydroxyapatite
CP	classical pathway
CR1	complement receptor type 1
Da	daltons
DAA	decay accelerating activity
DAF	decay accelerating factor
DDD	dense deposit disease
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FB	factor B
FD	factor D
FH	factor H
FHL-1	factor H-like protein
FHR	factor H-related protein
FI	factor I
GAGs	glycosaminoglycans
GBM	glomerular basement membrane
GPI	glycosylphosphatidylinositol
hFH	human factor H
HRP	horseradish peroxidase
K _D	equilibrium dissociation constant
LB	Luria Bertani
MAC	membrane attack complex
MASP	mannan-binding lectin-associated serine protease
MBL	mannan-binding lectin
MCP	membrane attack complex

MD	minimal dextrose
mFH	murine factor H
MPGN	membranoproliferative glomerulonephritis
OPD	o-Phenylenediamine dihydrochloride
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDI	protein disulphide isomerase
PMSF	phenylmethylsulfonyl fluoride
PspC	pneumococcal surface protein C
RCA	regulators of complement activation
rhFH	recombinant human factor H
rmFH	recombinant murine factor H
RU	response unit
SA	sialic acid
SCR	short consensus repeat
SDS	sodium dodecyl sulphate
SOC	super optimal catabolite suppressor
SPR	surface plasmon resonance
TED	thioester domain
TAE	tris-acetate buffer
TMB	tetramethylbenzidine
v/v	volume/volume ratio
w/v	weight/volume ratio
WB	Western blotting
YPD	yeast-peptone-dextrose
YPDS	yeast-peptone-dextrose-sorbitol
YPG	yeast-peptone-glycerol

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1: Introduction

1.1 The Complement system

1.1.1 Overview

The immune system has classically been divided into two components: innate (or non-specific) immunity; and adaptive (or specific) immunity. Historically these two arms were believed to operate independently, but it is increasingly recognized that there is extensive cooperation between them. Together they are responsible for eliminating invading micro-organisms as well as modified, and hence potentially pathogenic, host constituents (1, 2).

The complement system is an evolutionarily ancient molecular constituent of immunity that probably first appeared in a primitive form in simple organisms more than 1000 million years ago (3). The term 'complement' first introduced in the 19th Century, reflected its emerging role in 'complementing' the action of antibodies. The complement system is now known to be a highly integrated network of nearly 50 proteins that act in an orchestrated fashion. Complement is a powerful first line of innate defence (4), a bridge between innate and acquired immunity and a key player in the clearance of immune complexes and debris resulting from stressed, damaged or dying cells (5). The complement system is activated not just by antibody-antigen complexes but also directly by pathogen-associated or 'danger'-associated molecular patterns (1), or simply by a lack on a surface of host self markers. Following activation, complement can influence both B and T-cells leading to a state of immune vigilance (6). These functions are summarised in Table 1.1.

COMPLEMENT ACTIVITY	PROTEIN RESPONSIBLE
<i>Host defence against infection</i>	
• Opsonization • Chemotaxis and activation of leucocytes • Lysis of bacteria and cells	• Covalently bound C3b and C4b; receptors on erythrocytes and phagocytes • Anaphylatoxins (C5a, C3a, C4a); anaphylotoxin receptors on leucocytes • Membrane attack complex (C5b-C9)
<i>Interface between innate & adaptive immunity</i>	
• Augmentation of antibody responses • Enhancement of immunologic memory	• C3b and C4b bound to immune complexes and to antigen; C3-fragment receptors on B-cells and antigen presenting cells • C3b and C4b bound to immune complexes and to antigen; C3 fragment receptors on follicular dendritic cells
<i>Disposal of waste</i>	
• Clearance of immune complexes • Clearance of apoptotic cells	• C1q; covalently bound fragments of C3 and C4 • C1q; covalently bound fragments of C3 and C4

Table 1.1 The three main physiologic activities of the complement system. Adapted from (2) (5).

Complement activation can be initiated by the classical pathway (CP), the lectin pathway (LP) or the alternative pathway (AP) (Figure 1.1). Each pathway involves a proteolytic cascade resulting in rapid amplification and converging at cleavage of the protein C3. The conversion of C3 to C3a and C3b initiates the terminal pathway that results in release of C5a and the formation of the cytolytic membrane attack complex (MAC).

1.1.2 The classical pathway

The CP is activated by binding of the C1q component of the C1 complex of complement to antibody-antigen complexes. C1 is a molecular complex consisting of C1q associated with two molecules each of the proenzymes C1r and C1s. On binding to the Fc portion of antigen-bound IgG or IgM, a conformational change occurs in C1q that results in C1r cleaving and activating C1s. Activated C1s cleaves C4 to C4b that can attach to surfaces *via* a covalent bond. C4b binds the pro-enzyme C2 to form the pro-convertase complex C4bC2. In this context, C2 is also susceptible to cleavage by activated C1s, yielding C4b2a, which is termed the CP C3 convertase (7) and generates C3a and C3b. Some of the C3b molecules remain in solution, others can bind

covalently to surfaces (like C4b, C3b has an internal thioester that is activated and exposed upon C3 cleavage) and some bind non-covalently to C4b2a, forming C4b2aC3b. The presence of C3b here directs the specificity of the convertase towards C5, hence C4b2aC3b is termed the CP C5 convertase.

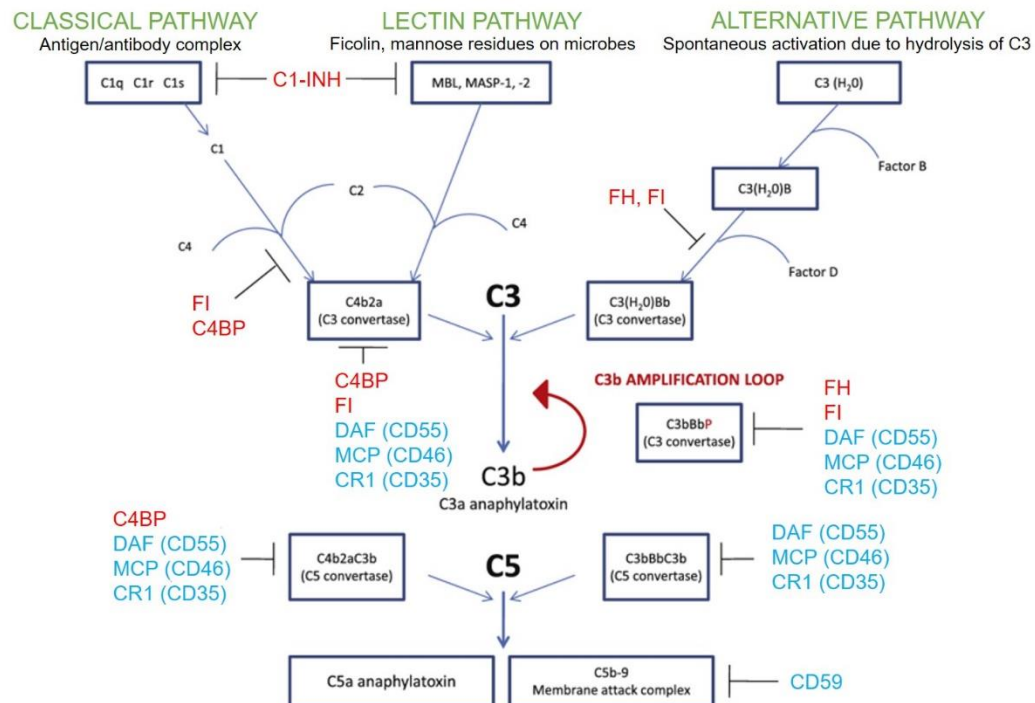


Figure 1.1 Schematic depicting the complement system. Diagram depicting the three pathways of complement activation, the C3b amplification loop and the terminal pathway including C5 activation. Complement regulatory proteins are in plasma (red text) or attached to cell surfaces (blue text). Properdin (P) stabilises the AP C3 convertase and is considered a positive regulator. MBL: mannose-binding lectin; MASP: MBL-associated serine protease; C1-INH: C1 inhibitor; C4BP: C4 binding protein; DAF: decay-accelerating factor (CD55); MCP: membrane co-factor protein (CD46); FH: factor H; FI: factor I. CR1: complement receptor 1 (CD35); P: properdin. Figure and legend adapted from (8).

1.1.3 The lectin pathway

The LP is activated by carbohydrate residues present on the cell surfaces of pathological organisms. Mannose-binding lectin (MBL), in association with the serine proteases mannose-binding lectin-associated proteases 1 and 2 (MASP 1 and MASP2) is utilized as the recognition protein, and is similar to C1 in the CP. MASP1 and MASP2 (in a similar manner to C1r and C1s of the CP) lead to the formation of C4b2a, i.e. the CP C3 convertase, and subsequent cleavage of C3 to C3b (7).

1.1.4 The alternative pathway

The AP is continually active albeit at a subdued level. In a so-called ‘tick-over’ process, the thioester of C3 spontaneously hydrolyses at a low rate, forming C3(H₂O). C3(H₂O) formation is linked to a structural change exposing the C3 thioester domain (TED) (9). C3(H₂O) resembles C3b, and it can bind the proenzyme factor B (FB) (10), forming C3(H₂O)FB. In this context, FB is cleaved by complement factor D (FD) to form enzymatically active C3b(H₂O)Bb, which is termed the initiating C3 convertase. This can cleave C3 into C3a and more C3b. As in the CP, C3b can remain in solution, or covalently attach to a hydroxyl group on any nearby surface. In the fluid phase and on host cells, specifically, surface-bound C3b is generally rapidly inactivated by dedicated complement regulatory proteins (see Section 1.1.6).

Nascent C3b (originating from either the AP or the CP) bound to foreign surfaces (as well as any residual C3b on host surfaces and in the fluid phase) binds FB to form a proconvertase (C3bB) which is cleaved by FD, to form the catalytically active convertase of the AP (C3bBb) (Figure 1.2) (11). Surface bound C3bBb is a short-lived complex, with a half-life of ~ 90 s (12), that can be stabilised by properdin. Properdin can also help to recruit C3b and C3bBb to host surfaces. C3bBb specifically cleaves C3 to generate additional molecules of C3b. If these molecules are generated close to the surface they will attach to the same surface in the immediate vicinity, thus seeding the formation of further C3 convertases in a positive-feedback loop. This results in rapid deposition of multiple molecules of C3b on foreign surfaces. On host surfaces, complement regulators (see Figure 1.1 and Section 1.1.6) not only inactivate C3b as mentioned above, but also block C3bBb formation and then shorten the lifespan of any C3bBb that does manage to form. C3bBb binds one or more additional C3b molecules to form the AP C5 convertase (C3bBbC3b).

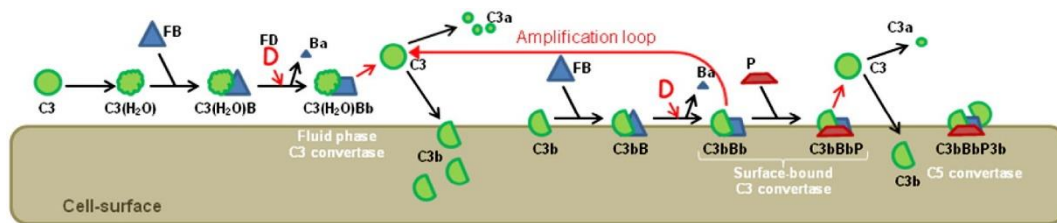


Figure 1.2 Formation of the alternative pathway C3 convertase. Two different C3 convertases are shown, the initiating C3 convertase ($C3(H_2O)Bb$) and the surface-bound C3 convertase ($C3bBb$). Formation of the latter convertase, which can be stabilised by properdin (P), completes the C3b amplification loop and can lead to the formation of the C5 convertase. Figure and legend adapted from (13).

1.1.5 Terminal pathway

In the absence of regulators, the C5 convertases (classical/lectin pathway: $C4b2aC3b$; alternative pathway: $C3bBbC3b$) cleave C5 into C5a and C5b. C5a (with C3a and C4a) are anaphylotoxins, capable of initiating an inflammatory response. C5b binds sequentially to C6, C7, C8 and multiple molecules of C9 to form the membrane attack complex (MAC). The MAC creates a membrane-penetrating pore leading to cell lysis (14).

1.1.6 Regulators of complement activation

Once triggered, activation of complement can occur very rapidly due to the C3b positive-feedback loop. Regulation of activation is essential to avoid damage to healthy host tissues. This is particularly important in the case of the AP due to “tick over” (see above). As will be seen (Section 1.4), deficiencies in complement regulation underlie diseases with significant morbidity and mortality.

Complement regulatory proteins can act both in fluid-phase and on the surfaces of cells or of extracellular matrix. Most are members of the regulators of complement activation family (RCA). These proteins mainly consist of numerous examples of one domain-type, the complement control protein (CCP) module (also known as sushi domain or short consensus repeat (SCR)). For example, complement FH consists entirely of 20 CCP domains. Regulation mechanisms include the prevention of convertase formation, promotion of convertase decay and proteolytic degradation of activated components (4).

Table 1.2 summarises the main regulators of the RCA family, while Figure 1.3 shows the involvement of regulatory protein in C3b degradation.

REGULATOR	LIGAND SPECIFICITY	FUNCTIONAL ACTIVITY
Fluid-phase regulators		
C1-INH	C1r/C1s	Inhibits C1r and C1s mainly in fluid phase
C4BP	C4b	Cofactor for factor I in cleavage of C4b; accelerates convertase decay (classical/lectin pathway)
Factor H	C3b	Cofactor for factor I in cleavage of C3b; decay acceleration of C3 convertase
Factor I	Factor H/C4BP/MCP/CR1	Cleaves C3b and C4b
Cell-membrane regulators		
DAF	C3b/C4b	Accelerates decay of C3 and C5 convertases
MCP	C3b/C4b	Cofactor for factor I in cleavage of C4b and C3b
CR1	C3b/C4b	Cofactor for factor I in cleavage of C4b and C3b; accelerates decay of C3 and C5 convertases
CD59	C5b-8/C9	Prevents assembly of cytolytic MAC on self cell

Table 1.2 Summary of the regulators of complement activation. C1-INH: C1 inhibitor; C4BP: C4 binding protein; DAF: decay-accelerating factor; MCP: membrane co-factor protein; CR1: complement receptor 1. Adapted from (15).

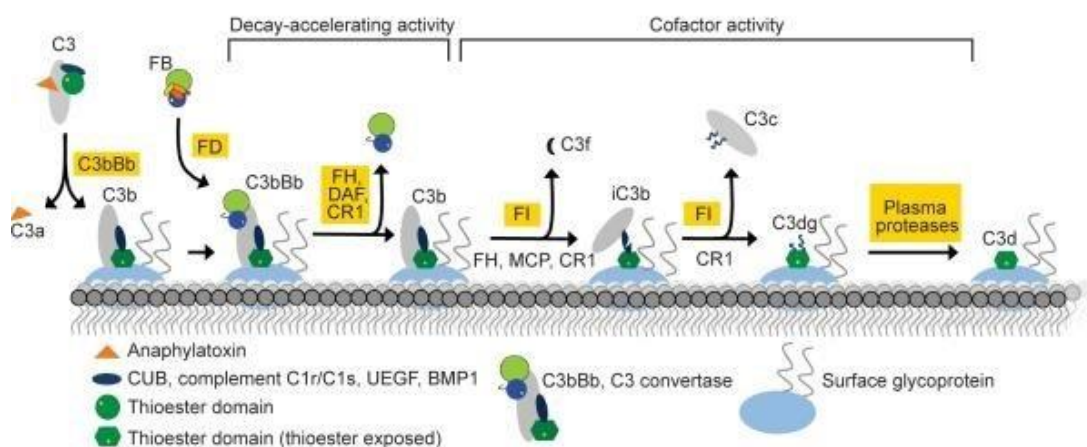


Figure 1.3 Degradation of C3b in the presence of complement regulatory molecules. In the presence of regulatory molecules, C3b may be degraded sequentially to iC3b, C3c, C3dg and C3d. C3d corresponds to the TED (thioester domain) and remains surface-bound on the cell surface. FB: factor B; FD: factor D; FH: factor H; FI: factor I; DAF: decay-accelerating factor; MCP: membrane co-factor protein; CR1: complement receptor 1. Figure and legend adapted from (16).

Factor H (FH) is the most important regulator of the AP, acting in both the fluid-phase and on cell surfaces. Given its importance and central role in my project, it will be discussed further in Section 1.2.

1.2 Factor H

Factor H (FH) is a 155-kDa single-chain soluble glycoprotein first identified in 1965 as β 1H globulin (17). The FH gene (*CFH*) is located on chromosome 1q32 in the RCA gene cluster, adjacent to the five genes that code for the factor H-related proteins (FHRs)(18). FH consists of 1213 amino acids, organised into 20 CCPs of ~ 60 amino acids each joined by very short linking sequences (19). Native FH contains eight diantennary disialylated glycans, known as N-glycans (out of nine potential sites) (Figure 1.4) (20). FH is produced primarily by the liver, and whilst previously thought to circulate at a concentration range of 116-562 $\mu\text{g/ml}$ (21), more recent studies have estimated 150-300 $\mu\text{g/ml}$ (22). Thus, normal FH concentrations in human plasma are approximately 1-2 μM .

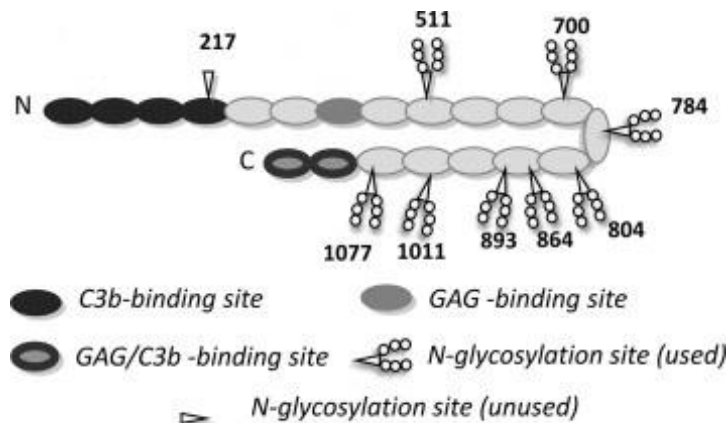


Figure 1.4 Summary of FH structure and binding sites. The cartoon drawing of factor H shows its 20 CCP modules as lozenge shapes (ovals). Shading signifies binding sites for C3b and glycosaminoglycans (GAGs) (or both in the case of CCP modules 19 and 20 that also bind to sialic acid). Eight of nine potential N-glycosylation sites are occupied (as indicated) in the native protein. Figure and legend adapted from (23).

1.2.1 Structure of FH

Each of the 20 CCPs of FH has four invariant cysteines (I-IV) along with a near-invariant tryptophan located in the sequence between CysIII and CysIV. Other highly conserved residues include glycines, prolines, and various hydrophobic residues (Figure 1.5) (24). This consensus sequence occurs frequently in other members of the RCA family, including C4BP, CR1, DAF,

MCP and FHR proteins (25) as well as many non-complement proteins. Each of the CCPs is presumed to fold into a similar three-dimensional structure (24), stabilized by Cys(I)-Cys(III) and Cys(II)-Cys(IV) disulphide linkages. A compact hydrophobic core is wrapped in anti-parallel β -sheets linked by loops and turns (26).

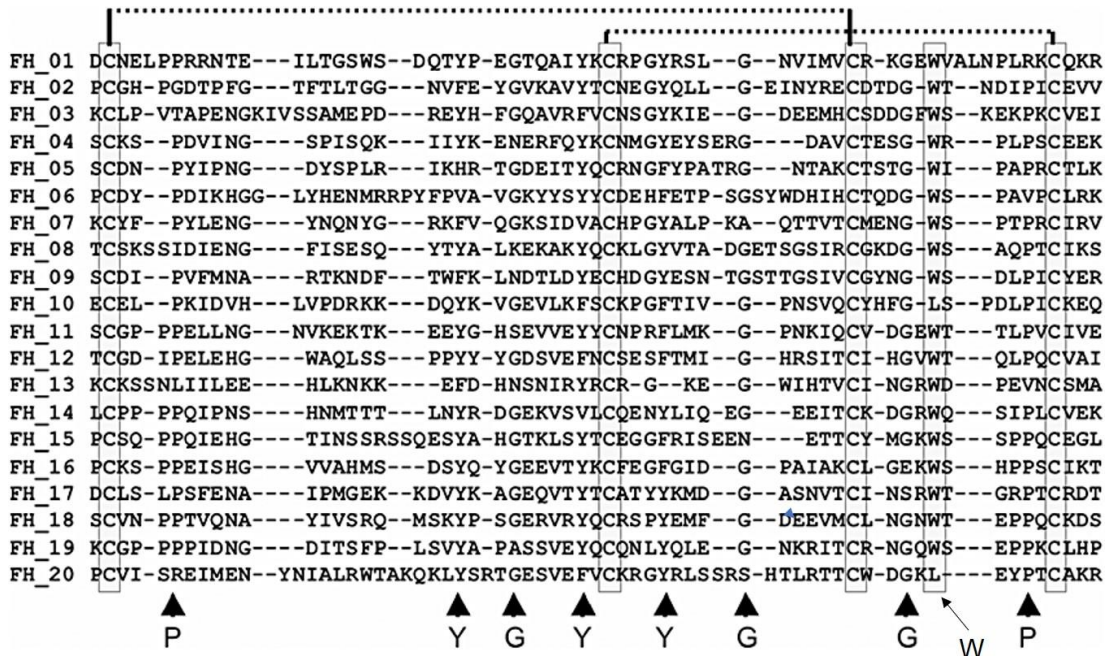


Figure 1.5 Multiple sequence alignment of 20 CCP modules in sequence of FH. One letter codes used throughout. Invariant Cys residues and almost invariant tryptophan residues are highlighted by boxes. Horizontal lines indicate disulphide bonds within CCP modules. Figure and legend adapted from (24).

1.2.2 Functions of FH

Factor H acts as a regulator of the AP in three well-characterised ways: i) It is a cofactor for the serine protease FI in cleaving C3b into the “inactive” form, iC3b (Figure 1.3) with release of the small fragment C3f (this is known as cofactor activity, CA) (27). iC3b remains an opsonin but cannot bind FB. Sequential cleavage by FI of the iC3b α' -chain (with other cofactors e.g. CR1) generates C3c that is released and C3dg (that can be further degraded by other proteases to C3d), which remains bound to the cell surface (10); ii) FH blocks assembly of the AP C3 (and C5) convertases by competing with FB for binding to C3b; iii) FH accelerates the decay of AP convertase, yielding C3b

and Bb that cannot re-associate (this is called decay-acceleration activity, DAA) (10, 28). All three of these regulatory actions of FH are modulated by the chemical make-up of the surface to which C3b is bound (see Section 1.2.3.2), and this is the basis for discrimination of the AP of complement between self and non-self.

1.2.3 Binding sites of FH

1.2.3.1 C3b-binding sites

Binding to C3b is key to the complement-regulatory functions of FH. The four N-terminal modules (CCPs 1-4) of FH form a key C3b-binding site and these modules are both necessary and sufficient for *in vitro* CA and DAA (29, 30). A crystal structure of a recombinant fragment of FH consisting of CCPs 1-4 in complex with C3b has been determined (29) showing a very extensive interface between the two molecules. A further binding site for C3b is formed by the two C-terminal modules (CCPs 19-20). These bind to the thioester domain of C3b, that corresponds to C3d (the fragment of C3 remaining after full cleavage). The crystal structures of C3d in complex with a recombinant fragment of FH consisting of CCPs 19-20 was determined (16, 31), which suggested that the main binding site for C3b is located in CCP 19 and the linker between CCPs 19 and 20. A further structure determination was reported for an engineered version of FH in which both CCPs 1-4 and CCPs 19-20 are bound to the same molecule of C3b. Note that there is no experimentally determined 3D structure, only models, for the FH/C3b complex.

A weaker C3b binding site might also exist in CCPs 6-8 (32).

1.2.3.2 Polyanion binding

Fundamental to the ability of FH to regulate the AP is its ability to bind strongly to C3b immobilised on a surface that requires protection from complement, whilst largely ignoring C3b on a bacterial surface. This selectivity is achieved, through interactions of FH with polyanions (33) such as sialic acids and glycosaminoglycans (GAGs). Pathogenic surfaces (e.g. of microbes) lack sialic acids and host-like GAGs, so do not allow any significant FH binding, and therefore complement activation can proceed unchecked. Heparin is used

as a model for host glycosaminoglycans, and functional binding studies have shown the major heparin binding sites to be in CCPs 7 (with contributions from 6-8 and 19-20) (34-36).

The C-terminal site seems to dominate self *versus* non-self discrimination. The recombinant fragment FH CCPs 19-20 (37) discriminates between host and non-host cells and can inhibit full-length FH from binding to cell surface-bound C3b, despite the presence of the C3b binding site in CCPs 1-4. In addition, monoclonal CCP-20 specific antibodies can also block interactions of FH with endothelial cells (38). The importance of CCPs 19-20 in the function of FH is illustrated by the fact that polymorphisms or mutations in this area may affect the engagement of FH with host cells and are implicated in disease, as will be discussed in Section 1.4.

1.2.4 FH protein family

The FH protein family consists of seven proteins, all composed from CCP modules: FH, Factor H-like protein 1 (FHL-1) and five factor H-related proteins (FHR-1, -2, -3, -4 and -5) (39, 40).

1.2.4.1 FHL-1

FHL-1 is a truncated splice variant of FH arising from the introduction of a stop codon. It is identical to the first seven CCPs of FH with an additional four-amino acid residue extension at its C-terminus. Due to the presence of FH CCPs 1-4 and 7 within FHL-1, this protein exhibits CA and DAA plus the abilities to bind C3b and heparin (40-42). CCP 7 of both FH and FHL-1 accommodate the Y402H polymorphism that predisposes to age-related macular degeneration (43). Despite their similarities, FH and FHL-1 are reported to have differing expression patterns (44), with FHL-1 having a distinct role in cell-adhesion (45).

1.2.4.2 Factor H-related proteins

The FHR genes in the RCA gene cluster arose as a result of genetic duplications resulting in the formation of several genes with a high sequence similarity to various sections of FH, named FHRs 1-5. (Figure 1.6). Although

exact plasma concentrations of the FHR proteins are not well characterised, they appear consistently lower than that of FH (46-50).

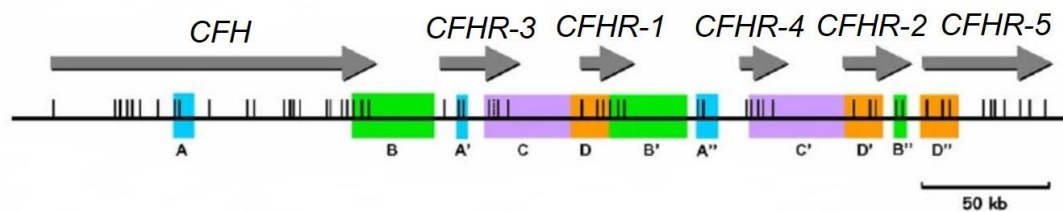


Figure 1.6 Genomic organisation of the *CFH* and *CFHR 1-5* genes. Arrows represent the genes with their names. The coloured boxes indicate the sequence repeats. The vertical lines indicate the position of the exons of the *CFH* and *CFHR* genes. Figure and legend adapted from (51).

The FHR proteins are composed of domains exhibiting varying degrees of amino acid sequence identity to each other and to FH, as illustrated in Figure 1.7.

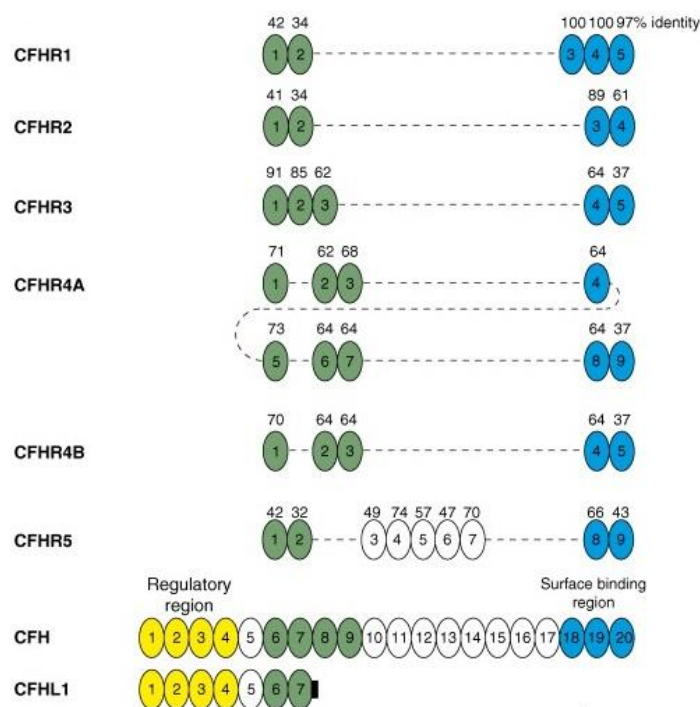


Figure 1.7 Human FH gene cluster and structure of related proteins. Homologous domains inferred from high sequence similarity indicated by vertical alignment. Numbers above each CCP indicating identity to the corresponding domain in *CFH* at the protein level. *FHR4* is present in plasma in two isoforms derived from the *CFHR4* gene by alternative splicing. Figure and legend adapted from (52).

Multiple studies have attempted to elucidate the functions of the FHR proteins. Given their homology to FH, it was initially proposed that FHRs would exhibit complement regulatory roles similar to FH (39). Indeed, despite none of the five FHRs containing CCPs that are similar to CCPs 1-4 of FH (Figure 1.7), several studies have suggested weak CA or DAA (53, 54) with attention focussed on FHR 3, 4 and 5. However non-physiological (high) concentrations of the FHRs were used in these assays (55). More recent studies (56, 57) suggest intriguingly that FHR proteins may compete with FH for C3b binding and hence prevent the regulatory activities of FH on surface-bound C3b. CCPs 1-2 of FHRs 1, 2 and 5 show high sequence similarity to each other and exhibit dimerization. Dimeric forms of FHRs could enable them to act as more effective competitive antagonists to FH, preventing its normal regulatory function and therefore 'deregulating' it (56). Further studies are required to delineate the ligands and surface-specificity of the FHRs.

Due to the high degree of sequence similarity, the *CFH-CFHR* gene region is prone to chromosomal recombination and duplication events in which whole genes or exons may be altered (51, 58). The most frequent event (reported frequency in the U.K. of 17.3% (59)) has resulted in a *CFH-CFHR* allele in which recombination led to complete loss of the *CFHR3* and *CFHR1* genes ($\Delta CFHR3-1$) that has been associated with atypical haemolytic uraemic syndrome (aHUS) (58). Other disease-associated rearrangements include a *CFH-CFHR1* hybrid gene in aHUS (60) and an internal duplication in the *CFHR5* gene in familial C3 glomerulonephritis (termed CFHR-5 nephropathy) endemic in Cyprus (61)(Section 1.4).

1.2.5 FH and immune evasion

There are several mechanisms for evasion of complement by pathogens. These include: recruitment of complement regulators, inactivation of complement proteins caused by enzymatic degradation, and inhibiting complement proteins by interacting with them directly (62, 63). Given the importance of complement and FH in protection against pathogens, it is unsurprising that many pathogens have evolved the ability to sequester host

complement regulators such as FH. The number of known pathogenic agents that utilise binding of FH grows ever larger. It includes *Streptococcus pneumoniae*, an important pathogen causing serious infections such as pneumonia, meningitis and septicaemia (64). One of the strategies employed by *S. pneumoniae* for escaping the immune system is to express a virulence factor: the surface protein PspC which binds to FH and minimizes C3b deposition on pneumococci (65). This will be further explored in my project in the context of recombinant human FH.

1.3 Murine complement system

The development of murine models of disease underpins much immunological research, with potential new therapies often first tested in a murine model for the target disease. Translating results to human conditions however requires an understanding of both the similarities and differences between the human and mouse complement system, which will be delineated below.

1.3.1 Membrane complement regulatory proteins

The RCA gene cluster between humans and mice is well conserved. In humans the RCA cluster can be split into two regions, the first encompassing CFH and the CFHRs, the second containing the genes encoding C4BP, DAF, CR2, CR1 and MCP. In mice, the RCA cluster is composed of three regions, the first containing CFH and related proteins, the second encoding C4BP and two DAF variants, and the third containing CR 1-related protein y (Crry), CR2 and MCP (66).

1.3.1.1 DAF

Mice contain two separate DAF genes, DAF-1 and DAF-2. The DAF-1 gene encodes a GPI-anchored protein that is expressed ubiquitously in several mouse tissues, whereas DAF-2 encodes a transmembrane protein that is selectively expressed in the testis (67). Therefore the GPI-anchored DAF-1 can be considered to be equivalent to human DAF. As in humans, murine DAF functions as a C3 convertase inhibitor (68).

1.3.1.2 MCP

A difference exists between human and murine MCP in their tissue expression patterns. Whilst human MCP is widely distributed, MCP in the mouse is only expressed in the testis (69).

1.3.1.3 CR1

In humans, CR1 and CR2 are encoded by distinct but closely linked loci on chromosome 1 (70). CR1 is expressed on B-cells, follicular dendritic cells (FDC), erythrocytes, polymorphonuclear cells and phagocytic macrophages. In mice, CR1 and CR2 are encoded by a single gene, *mCr2*, at the RCA locus (66). Murine CR1 is mainly expressed on B-cells and FDC, but not on erythrocytes or platelets (71), implying murine CR1 may have a less important role as an immune adherence receptor than its human counterpart.

FH on the surface of platelets and podocytes in rodents appears to be involved in the processing of immune complexes through binding to C3b in the complexes, in an analogous manner to CR1 on erythrocytes and podocytes in primates (72).

1.3.1.4 CD59

CD59 is encoded for by two genes (in chromosome 2), CD59a and CD59b (73). CD59a is more broadly expressed in mice with CD59b being predominantly expressed in the testis.

1.3.1.5 Crry

Crry (see above) is a transmembrane protein not present in humans that was initially isolated in mice using human CR1 cDNA as a probe, and was therefore termed CR 1-related gene protein y (74). Crry regulates C3 activation in the same way as human MCP and DAF, acting as a cofactor for the FI-mediated inactivation of mouse C3b and C4b and possessing DAA for the CP and AP C3 convertases (75, 76). It cannot act as a receptor for mouse C3b-coated erythrocytes, despite being structurally related to CR1. Like human DAF and MCP, mouse Crry is widely expressed in many tissues (77). Crry deficiency is embryonically lethal as a result of the failure to regulate maternal complement

activation at the foetal-placental interface, although viable Crry-deficient animals can be generated if the mother is rendered C3 deficient (78). It has previously been shown that Crry-expressing cells *in vitro* are protected from C3b deposition (76). Crry is therefore critical in the mouse as it is the only ubiquitously-expressed membrane-bound regulatory molecule.

1.3.2 FH protein family

1.3.2.1 Murine FH

Murine FH (mFH) (UniProtKB - P06909) is a soluble single-chain polypeptide glycoprotein consisting of 1234 amino acid residues (including an 18-residue signal sequence). These form 20 CCPs, each ~ 61 amino acids in length (79). The glycosylated form of human (h)FH is 155 kDa, whereas the molecular weight of glycosylated mFH is unknown, but it migrates at the same apparent molecular weight as hFH on SDS PAGE (80). Murine FH and hFH show 63% sequence identity, whilst individual CCP domains can show up to 78% sequence identity (Figure 1.8). While hFH has nine potential N-glycosylation sites, eight of which are occupied (20), mFH has seven putative glycosylation sites, with five confirmed as occupied (79). Although the position of glycosylation sites differ, both proteins are predominantly glycosylated in the C-terminal half of the protein.

At least two allotypes of mFH have been reported (mFH.1, mFH.2) (81) which are thought to be functionally equivalent (82). mFH-1 is the predominant allotype in laboratory-strain mice (81). Firm data on the concentration of mFH in the plasma of mice is not available, although Ding *et al* (83) reported a value of ~ 200 µg/ml mFH in mice of C57 lineage, which is within the range of hFH (22). No equivalent to FHL-1, the splice variant of hFH, has been detected in mice.

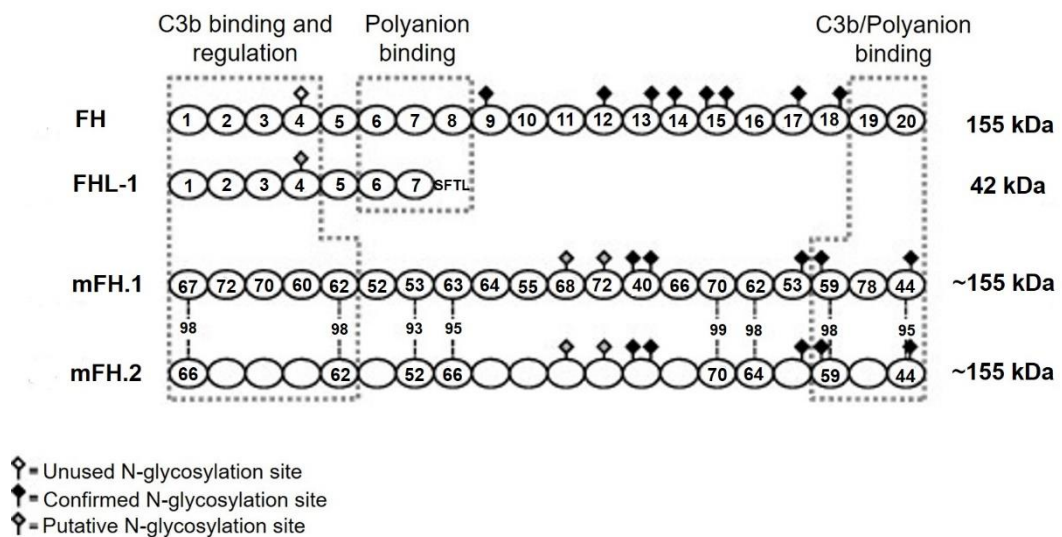


Figure 1.8 Comparison of human and murine FH sequence. Schematic representation of the 20 CCP modules of human FH, FHL-1 and the two common mouse *Cfh* allotypes, mFH.1 and mFH.2. Confirmed N-linked glycosylation sites as well as putative or confirmed unused sites are shown. Numbers within each CCP module of mFH.1 and mFH.2 represent the percentage of identical residues compared to the equivalent module in human FH (modules of mFH.2 that do not contain any number are identical to the corresponding module of mFH.1). Numbers between mFH.1 and mFH.2 indicate the percentage identity between the indicated modules. Regions identified as having equivalent functional properties are outlined. Figure and legend adapted from (84).

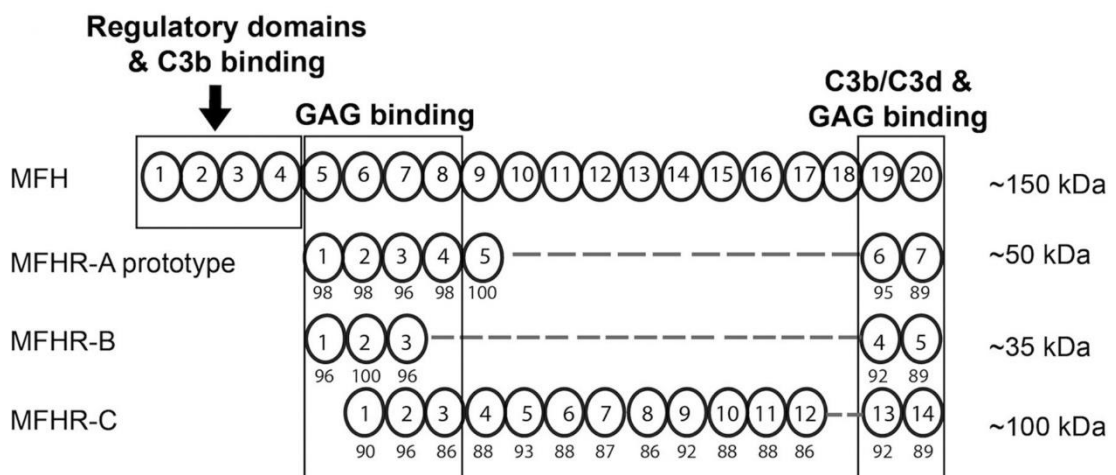


Figure 1.9 Schematic representation of the CCP modules of mFH and mFHR proteins. Both mFHR-B and mFHR-C proteins have been detected in plasma. mFHR-A is a predicted protein based on its gene (85). The mFHR proteins are shown with CCP domains aligned with CCP domains of mFH, with percentage homology with mFH shown below each CCP. Figure and legend adapted from (86).

Using recombinant mFH fragments (CCPs 1-5 and 18-20), it was confirmed that both mFH and hFH utilise similar regions for binding C3b and for CA and DAA (Figure 1.8) (80).

1.3.2.2 Murine FH-related proteins

Like humans, mice have five mFHR genes, and four mRNA transcripts were identified from a mouse liver cDNA library (85) implying at least four mFHR proteins are produced. Hellwage et al (87) expressed two mFHRs recombinantly (mFHR-B and mFHR-C) and characterised them as heparin and C3b binding proteins. These two putative mFHR proteins show even higher sequence identity to mFH than do hFHRs to hFH (Figures 1.7, 1.9). This may suggest that the mFHRs are not orthologues of hFHRs. Notably, mFHR-B and mFHR-C do not contain the dimerization motif found within hFHR-1, hFHR-2 and hFHR-5 (56). Like hFHRs, mFHRs lack equivalent CCP modules to the regulatory CCPs 1-4 of mFH and hFH.

To further characterise mFHRs, Antonioli et al (86) expressed recombinant versions of mFHR-B and mFHR -C, proteins that have been reported in mouse plasma, as well as the inferred (from the sequence of its transcript) protein mFHR-A (Figure 1.9). MFHR-A and -B were found to be potent antagonists of mFH on sheep erythrocytes and bound C3d (K_D s were in order of decreasing value, mFHR-A > mFHR-B > mFHR-C > mFH). MFHR-A and -B were also found to antagonise the effect of mFH on renal tubular epithelial cells, increasing complement activation as indicated by an increase in C3b deposition. It may be therefore that mFHRs have equivalent complement 'deregulatory' functions to hFHRs. The function of mFHR-C has yet to be fully elucidated. Understanding how these proteins function in different contexts (e.g. fluid-phase versus the cell surface) is important when assessing potential FH-based therapeutics in murine models of human disease.

1.4 FH and renal disease

Given its importance as a regulator of the alternative pathway, it is not unexpected that mutations or deficiencies in FH are implicated in disease. My

project is focussed on recombinant factor H and two rare renal diseases, atypical haemolytic uraemic syndrome (aHUS) and C3 Glomerulopathy (C3G) which will be discussed further here, along with the relevant murine models of disease.

1.4.1 Atypical haemolytic uraemic syndrome

Atypical HUS is a rare disease characterized by acute kidney injury, thrombocytopenia and microangiopathic haemolytic uraemia (88), with a reported incidence of 0.4 per million per year (89).

Historically, the term aHUS was used for all thrombotic microangiopathies (TMA) not found to be caused by shiga-toxin producing *Escherichia coli*. Current classifications have evolved with an increased understanding of disease mechanisms, and the term 'Primary aHUS' is used when an underlying abnormality of the AP of complement is suspected and other secondary causes (e.g. drugs, infection, glomerular disease, malignancy, post-solid organ transplant) are excluded, even though an underlying complement abnormality may not be identified (90).

Atypical HUS is considered a 'thrombotic microangiopathy', the pathological features of which represent tissue response to endothelial injury, namely endothelial swelling and mesangiolysis in active lesions, and double contours of the basement membrane in chronic lesions. However, in some renal biopsies overt thrombosis, as evidenced by intra-luminal fibrin or fibrin-platelet plugging, is not seen. It has therefore been proposed that the pathological process is more accurately described as microangiopathy +/- thrombosis (91).

1.4.1.1 Genetics

Mutations in FH in aHUS were first described over 20 years ago (92), and account for up to 25% of cases (93), making them the commonest cause of complement-mediated aHUS. The majority of aHUS-linked FH mutations occur in the C-terminal domains of FH, the region that mediates binding to self-surface *via* GAGs and surface-attached C3b (94). A large number of studies (95-99) have examined FH mutations in this region, either by purifying the

mutant FH from plasma, or by testing mutant recombinant FH fragments in CCPs 19-20. The disease-linked mutations have variable effects on binding to C3b and GAGs/sialic acids, but many impair cell-surface complement regulation whilst maintaining normal complement regulatory activity in fluid-phase. The mutations are usually heterozygous and are regarded as predisposing rather than causative. The penetrance of the disease is age-related, with greater penetrance at older age (90). Later presentation of disease is more likely to be associated with a trigger such as infection, malignancy or pregnancy that activates complement, unmasking a latent defect (91).

As previously discussed, the location of the FH gene in the RCA cluster makes it prone to genomic rearrangements. The FH mutations S1191L, V1197A, and combined S1191L/V1197A arose through gene conversion between *FHR-1* and *FH* genes (51). Hybrid (fusion) proteins have also been generated (60) which lack either one or both of the 19-20 domains, thus also resulting in loss of cell surface regulation.

Mutations in several other regulatory complement proteins can predispose to aHUS. These include loss of function in other complement regulators, such as FI and MCP. The FI mutations are all heterozygous (100), comprising around 10% of complement-mediated aHUS, and result in loss of complement regulation of AP and CP (101). Mutations in MCP are the second most common cause of complement-mediated aHUS, accounting for ~ 15% of cases (102). Unlike FH, most MCP mutations result in a quantitative defect in MCP (88). Mutations in activation proteins of the AP such as C3 or FB are also found, resulting in gain-of-function. Mutations in C3 account for ~ 2-10% of complement-mediated aHUS cases, either by decreasing the affinity of C3b for its regulators, or increasing affinity of C3b for FB (103). Mutations in FB are rare (1-4% of cases), but lead to gain-of-function by enhanced convertase formation and resistance to convertase decay by regulators (104).

Anti-FH antibodies have been linked to acquired FH dysfunction (105) as they are directed against the surface recognition domains (106). They are most

frequently seen in patients with a homozygous deletion of *CFHR3/CFHR1*, and aHUS in this setting is termed DEAP-HUS (deficiency of FHR proteins and autoantibody positive-HUS).

1.4.1.2 Treatment

Historically aHUS carried a very poor prognosis. Typically at 3-5 years after onset, 36-48% of children and 64-67% of adults have either reached end-stage renal disease or death (91). Patients with MCP mutations carry the best prognosis, whilst those with FH loss of function or gain-of-function in FB or C3 mutations carry the poorest prognosis. The only treatment was plasma exchange, but frequently the aHUS would become resistant over time, leading to end-stage renal disease and a life on dialysis or death (107). Renal transplants frequently failed, the outcome dependent on the underlying genetic abnormality, with only MCP mutations having a low recurrence risk post-transplant (108).

The use of Eculizumab, a recombinant humanized monoclonal antibody against C5 which blocks the cleavage of C5 into C5a and C5b, has now become the gold-standard treatment for complement-mediated aHUS. It is effective in both plasma-resistant and plasma-sensitive aHUS and its effect does not appear to be dependent on the type of underlying complement mutation (109). Currently it seems that for those patients who are successfully treated with Eculizumab, the treatment will need to be life-long. A small subset of patients do not appear to show improvement and have been found to have a thrombotic microangiopathy caused by non-complement genes or a rare C5 polymorphism which prevented Eculizumab binding (91).

1.4.1.3 Murine models of aHUS

Mice were genetically modified to produce a version of mFH, FH Δ 16-20, lacking the five C-terminal CCP modules. Since it is missing the key surface-recognition region found in CCPs 19-20 (Figure 1.8) (110), FH Δ 16-20 would be expected to have diminished surface recognition but to retain complement regulating functionality in fluid-phase (*i.e.* in plasma). These mice were crossed with FH-deficient mice (*CFH*^{-/-}) to generate homozygous (*CFH*^{-/-})

.FHΔ16-20) individuals. Higher C3 levels were observed in the plasma of *CFH*^{-/-}.FHΔ16-20 mice compared to *CFH*^{-/-} mice, confirming that FHΔ16-20 regulates plasma C3. The deposition of C3 along the glomerular basement membrane seen in *CFH*^{-/-} mice was also ameliorated by the presence of FHΔ16-20. As the *CFH*^{-/-}.FHΔ16-20 mice aged many developed haematuria and premature mortality with evidence of renal dysfunction and red cell fragments on blood films. The renal pathology showed changes consistent with TMA rather than the changes of C3 glomerulopathy seen in *CFH*^{-/-} mice. When *CFH*^{-/-}.FHΔ16-20 mice were crossed with C5-deficient mice, renal thrombotic microangiopathy was prevented (although abnormal mesangial C3 deposition was still seen) (111), giving an effect in mice similar to that seen in humans treated with Eculizumab.

Interestingly, similar to the variable disease penetrance in humans with complement gene mutations, there was also variable disease severity amongst *CFH*^{-/-}.FHΔ16-20 individuals, with aHUS not developing in 8/26 mice followed for four months, and 3/8 mice followed for eight months (110).

A second transgenic line produced FHΔ16-20 at 2% of normal wild-type levels. These animals did not develop TMA but did develop mild C3 glomerulopathy (110). This implies that some degree of complement regulation in plasma is required, in addition to defective regulation on the renal endothelium, in order for TMA to develop.

1.4.2 C3 Glomerulopathy

C3 glomerulopathy (C3G) describes a group of diseases in which uncontrolled complement activation can lead to complement protein deposition within the glomerulus (112). The diseases DDD (dense deposit disease), C3GN (C3 glomerulonephritis) and CFHR5 nephropathy are collectively known as C3 glomerulopathy (Figure 1.10). These diseases are rare, affecting ~ one person per million of population (113).

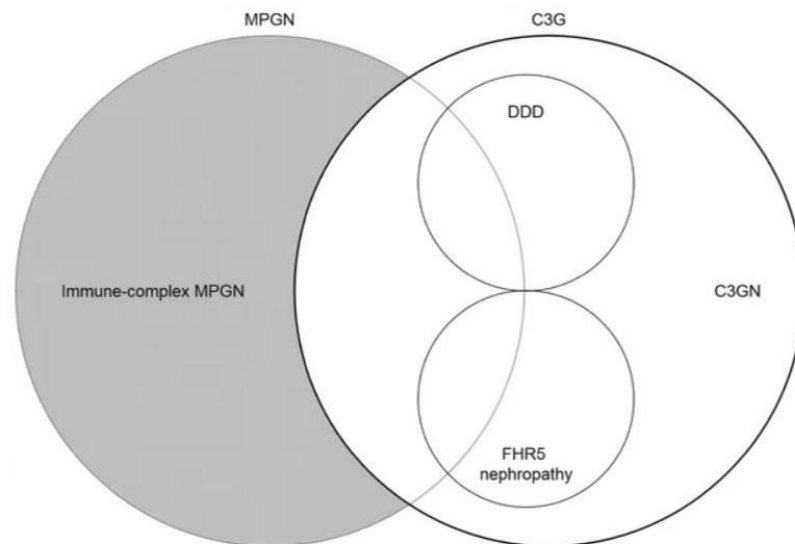


Figure 1.10 Schematic showing overlap of C3G and MPGN. A cause of uncontrolled complement activation should be considered in cases of C3 glomerulopathy (bold circle). Uncontrolled complement activation has also been identified in cases of immune-complex MPGN (shaded circle) – these are cases of MPGN that do not fit the criteria for C3G. Figure and legend adapted from (91).

1.4.2.1 Classification and pathology

Membranoproliferative glomerulonephritis (MPGN) is a pathological term describing a pattern of injury seen on light microscopy. It is characterised by mesangial expansion, cellular proliferation and double contouring of the basement membrane (114). The classification of MPGN was historically based upon the position of electron dense deposits relative to the GBM. There were three types: type 1: subendothelial deposits; type 2 (DDD) intramembranous deposits; and type 3: subendothelial and subepithelial. Types 1 and 3 generally stained positive for immunoglobulins and C3 on immunofluorescence (IF) whereas type 2 stained for C3 only (115). All three types were associated with uncontrolled complement activation and low C3 levels.

The term C3G was introduced to alert the clinician to the possibility of an underlying abnormality in the complement system (112). It is a histopathological diagnosis defined by the presence in renal biopsy samples of a glomerulonephritis with dominant glomerular IF staining of C3 at least two

orders of magnitude more intense than any other immune reactant. The presence of IgG does not preclude C3G.

DDD and C3GN can be distinguished based on their appearances under electron microscopy. DDD is defined by the presence of intramembranous electron-dense transformation of the GBM (116) and was historically termed MPGN-type II. However only about one third of renal biopsies have evidence of an MPGN pattern of injury; 45% have a mild mesangial hypercellularity, with other patterns including a crescentic or an acute proliferative and exudative pattern (117). DDD should therefore be considered as a form of C3G and not MPGN. Cases of C3G that do not fit the criteria of DDD are classed as C3GN.

C3GN is characterised by subendothelial electron-dense deposits and an absence of the highly electron-dense intramembranous change seen in DDD (113). Like DDD, light microscopic changes are heterogenous.

Figure 1.10 highlights the overlap between MPGN and C3G (91). In cases of MPGN without dominant C3 deposition, an underlying secondary cause should first be considered e.g. autoimmune disease, infections or paraproteinaemias.

1.4.2.2 Genetics

Genetic testing has shown that ~ 25% of patients with C3G have variants in complement-related genes (118), although these are not as well defined as in aHUS. Of importance to my project, FH deficiency leads to uncontrolled activation of AP in the fluid phase (119-121). FH deficiency can be quantitative (*i.e.* less protein is circulating) or functional (*i.e.* mutations lead to a functional impact such as defective binding to C3b, resulting in impairment of fluid-phase complement activation (122). Single nucleotide polymorphisms (SNPs) in FH have been studied in the context of MPGN/C3G, revealing that the Y402H polymorphism (123) is associated with increased risk of DDD, whereas the V62I polymorphism has been shown to be protective against DDD (124).

Other rare mutations in C3 or FB may be involved in the pathogenesis of C3G, including a C3 that forms a C3 convertase resistant to decay by FH (125). As

previously discussed (section 1.2.4.2), genomic duplications in the FHR region can cause disease *e.g.* CFHR-5 nephropathy (61).

However, most complement dysregulation in C3G is driven by autoantibodies. These can be to FH, C3 or FB, but the most frequently identified autoantibodies target C3bBb and are known as C3 nephritic factors. They stabilise the C3 convertase, thereby increasing its half-life which leads to increased consumption of C3 in serum. There is a reported prevalence of up to 80% in DDD and 50% in MPGN/C3GN (118). In addition, there is a recognized association of MPGN with other autoimmune diseases, including a substantially increased rate of Type 1 diabetes mellitus in relatives of patients with DDD (126).

Recent work (127) involved large-scale whole-genome sequencing of patients with Primary MPGN and C3GN to fully characterise the genetic factors contributing to these diseases. Importantly, no significant enrichment of rare variants in complement genes among cases was shown compared with controls, but the HLA locus was strongly associated. HLA serotypes DQ2 and DR17 were implicated; these genes encode components of the MHC Class 2 molecule that are found on the surface of antigen-presenting cells and are central to the initiation of the adaptive immune response and antibody production (128). This implies that rather than these diseases resulting from a primary genetic disorder of complement AP regulation, they may in fact be autoimmune in origin.

1.4.2.3 Clinical outcomes and treatment

The presentation of C3G ranges from asymptomatic haematuria and proteinuria to an acute presentation with the classic signs and symptoms of glomerulonephritis (113). They are typically, especially the DDD subset, diseases of childhood and young adulthood. Renal failure is progressive with 40% developing end-stage renal disease at 10 years (118). Plasma C3 levels are often low. Recurrence post-renal transplant is frequent, up to 80-90% in DDD (129).

The presenting features of patients with CFHR-5 nephropathy are a little different. First reported in large pedigrees of Cypriot ancestry, these patients often have synpharyngitic haematuria and may have previously been erroneously diagnosed as IgA nephropathy. Prognosis appears to be worse in males (61).

There are no universally effective treatments, due in part to the heterogenous nature of C3G. Treatments tried have included immunosuppressants such as steroids, cyclophosphamide and mycophenolate mofetil, but studies had small numbers of individuals and individual genetic and complement data was not available for most (113). Plasma exchange had mixed success (130) and again is likely dependent on the underlying complement abnormality.

Given the success of Eculizumab in aHUS it was hoped it would work similarly for C3GN. One clinical trial is reported to be underway but no data have been published as yet (91). Case reports have shown some success (131-133), mostly for patients with rapidly progressive disease and where prominent terminal pathway activity is seen. This mixed success is not unsurprising, given that, depending on the underlying complement abnormality, C3 convertase assembly and C3 fragment deposition would continue unchecked (134).

1.4.2.4 Animal models of C3G

Several animal models have been developed that assist in understanding the pathogenic disease progress in C3GN (Table 1.3). However the first reported animal (at the time termed MPGN Type II) was in a breed of Norwegian piglet that had developed spontaneous FH deficiency due to a mutation in CCP 20 that affected FH secretion (135-137). The animals died within 72 days from renal failure associated with a rapidly progressive glomerulonephritis. Histological examination revealed extensive dense deposits within a thickened GBM associated with capillary wall deposition of complement proteins but not immunoglobulin. This lesion was referred to as porcine DDD. The animals had low plasma C3 levels that were believed to reflect excessive fluid-phase complement activation.

CFH-deficient mice, *CFH*^{-/-}, were developed by targeted gene deletion of exon 3 of the murine FH gene in embryonic stem cells (119). These mice developed albuminuria with severely reduced plasma C3 levels. Light microscopic changes included mesangial hypercellularity and double contouring of the GBM. Electron microscopic changes included subendothelial electron dense deposits with mesangial cell interposition and new basement membrane formation, accompanied by capillary wall and mesangial C3 and C9 deposition. No IgG was seen. Deposits of C3 and C9 preceded the appearance of the subendothelial deposits, which preceded the light microscopic findings. Administration of serum-purified mFH (138) or hFH (139) restored plasma C3 levels and ameliorated C3 deposition in the glomerulus. Human FH was administered daily for ten days but this resulted in the development of anti-FH antibodies and likely immune-complex deposition in the glomerulus.

Animal model	Phenotype	Renal pathology	FH levels	C3 levels
CFH ^{-/-} pig	C3G	GBM thickening; mesangial cell proliferation	None	Low
CFH ^{-/-} mouse	C3G	Glomerular C3 staining on IF; C3GN	None	Low (30 µg/ml)
CFH ^{-/-} CFB ^{-/-}	Normal	No evidence of C3GN	None	normal
CFH ^{-/-} C5 ^{-/-}	Mild C3GN	C3GN less pronounced than CFH ^{-/-} mice	None	ND
CFH ^{-/-} P ^{-/-} mouse	C3G	Enhanced glomerular injury with increased glomerular C3 activation	None	Low (17 µg/ml)
CFH ^{m/m} mouse	C3G	Mesangial proliferation, hypercellularity, GBM thickening	Low (FH 1-18)	Low intact C3; high activated C3
CFH ^{m/m} P ^{-/-} mouse	Lethal C3G	Enhanced glomerular injury with DDD-like disease and increased glomerular C3 activation	Low (FH 1-18)	Low intact C3; high activated C3
FI ^{-/-} mouse	Mild kidney injury	Mesangial C3 staining on IF	Low (62% of WT)	Low (163 µg/ml)
CFH ^{-/-} FI ^{-/-}	Mild kidney injury	Mesangial C3 staining on IF; no evidence of C3GN	None	As FI ^{-/-}
Hepatocyte-FH ^{-/-} mouse	Mild kidney injury	Minimal glomerular hypercellularity	Low (19% of WT)	Low (42 µg/ml)

Table 1.3 Animal models of C3 glomerulopathy. References in main body of text. Figure adapted from (84, 113, 140).

Mice that were deficient in both FH and FB (*CFH*^{-/-}*FB*^{-/-}) (119) had normal plasma C3 levels and normal renal histology. The lack of FB meant the AP could not be activated, and therefore uncontrolled C3 activation, leading to the development of the C3GN lesion, did not occur.

A further *CFH*^{-/-} model was developed with additional C5 deficiency (*CFH*^{-/-}*C5*^{-/-}) (141). These mice had lower mortality and creatinine levels than *CFH*^{-/-} mice although they still have C3GN renal pathological changes, albeit less severe than in *CFH*^{-/-} mice.

CFH^{-/-} mice that are also properdin deficient (*CFH*^{-/-}*P*^{-/-}) (142) show exacerbated renal injury compared to *CFH*^{-/-} mice alone, thought to be due to favoured dysregulation activity of the C3 convertase over C5 convertase activity.

CFH^{m/m} mice (143) were generated that have a low level of a truncated FH protein, lacking CCPs 19-20. These mice show the characteristics of C3GN, although they have a higher plasma C3 level than *CFH*^{-/-} mice with a different C3 glomerular staining pattern, likely due to the residual FH functioning as a fluid-phase regulator. When these mice were crossed with a properdin deficient strain (*CFH*^{m/m}*P*^{-/-}) a lethal version of C3GN was found, with early onset proteinuria and death from crescentic glomerulonephritis by 10-12 weeks of age. When these mice were treated with a C5 inhibitor it was effective in preventing severe disease, and it also prevented death in 50% of the mice that had already developed severe active disease.

A hepatocyte conditional *CFH*^{-/-} mouse showed plasma FH levels of ~ 20% of WT (144). These mice do not show accumulation of C3 fragments along the glomerular basement membrane although they do have lower plasma C3 levels than WT mice. Interestingly, these mice develop a C5-dependent TMA after induction of complement activation within the kidney, rather than C3GN.

FI^{-/-} mice do not develop the C3GN changes observed in *CFH*^{-/-} mice, although they do show some evidence of mild kidney injury (145). *CFH*^{-/-}*FI*^{-/-} mice show no evidence of C3GN on light microscopy, with mesangial C3 glomerular deposition only. This rescuing of the C3GN phenotype by additional FI deficiency in *CFH*^{-/-} mice was interpreted to indicate that C3 cleavage products (e.g. iC3b) generated by FI, are essential for C3G pathogenesis in *CFH*^{-/-} mice.

The *CFH*^{-/-} mouse is the model used in my project. These mice were initially described as a murine model of DDD, although the mice develop subendothelial electron-dense deposits compared to intramembranous deposits in humans. Deposits were not seen frequently in the mesangium and did not appear in the basement membrane of the tubules and Bowman's capsule as seen in human DDD. They are therefore more appropriately described as a model of C3GN.

1.5 Aims of project

The search for a successful treatment for C3GN is ongoing. The inherent difficulties in purifying large volumes of murine FH from serum, and the development of anti-hFH antibodies with administration of hFH to *CFH*^{-/-} mice has precluded prolonged evaluation of FH treatment in *CFH*^{-/-} mice as a model for recombinant factor H replacement in patients with C3GN. Recombinant mFH produced in yeast is essential to test the idea of using recombinant hFH produced in yeast to treat (systemically) humans. Yeast-produced hFH is the best production system due to the quantities (kgs) that would be needed for any human trial in the future.

As an alternative to FH-replacement therapy, it might be possible to overcome a haploinsufficiency of FH by augmentation. PspCN (a small protein derived from a bacterial virulence factor) binds tightly to wild-type hFH and enhances its regulatory effectiveness (see Section 1.2.5). The effect of PspCN on disease-linked FH mutants requires further investigation.

I therefore aim to:

1. Generate, biophysically and functionally characterise full-length recombinant murine factor H (rmFH)
2. Administer rmFH to prevent development of C3GN in *CFH*^{-/-} mice
3. Administer rmFH to ameliorate renal injury due to C3GN in *CFH*^{-/-} mice
4. Produce full-length mutant variants of rhFH and characterise their function in conjunction with PspCN

Recombinant human FH (rhFH) has been previously produced and shown to be functional with *in vitro* assays in our laboratory using the yeast *Pichia pastoris* (23). Recombinant hFH has been produced in moss (146), mammalian (95), and insect cells (147) but yielded protein amounts inadequate for full characterisation, and orders of magnitude below therapeutic usefulness. The *P. pastoris* system will therefore be used for rmFH production with the aim that the volumes required for the proposed experiments (~ 500 mg) can be achieved.

2: Materials and Methods

2.1 DNA amplification, cloning, screening and *E. coli* transformation

2.1.1 Re-cloning recombinant murine factor H cDNA into pPICZαB

Recombinant murine factor H (rmFH) production using the vector pPIC3.5K (GeneArt, vector map Appendix B) may have failed in a previous attempt (by Fern Slingsby in the Barlow lab) due to the choice of secretion signal. It was therefore decided to try with the yeast alpha factor-secretion signal that had been previously used for the successful production of full-length recombinant human factor H (rhFH). Hence the first step was to excise the expression-optimised DNA encoding rmFH from the pPIC3.5K vector, and re-clone it into the pPICZαB vector that was used in the production of rhFH (23).

An *E. coli* stock containing the rmFH-encoding pPIC3.5K plasmid, which includes an ampicillin-resistance marker, was grown overnight at 37 °C on Luria-Bertani (LB) agar plates containing 50 µg/ml ampicillin. A single colony was subsequently grown overnight in 5 ml of LB medium containing 50 µg/ml ampicillin at 37 °C. Cells were spun down at 4000 g in a bench-top micro-centrifuge (Eppendorf) for 15 minutes, then the plasmid was extracted from the pellet using a QIAprep Spin Miniprep kit (Qiagen) according to the protocols provided by the manufacturer.

2.1.1.1 Amplification of DNA

The polymerase chain reaction (PCR) was used for the amplification of DNA sequences, screening of colonies and sequencing of clones. All primers used were ordered from Invitrogen and shipped de-salted.

Primers were designed to engineer in *Pst*I and *Xba*I restriction sites at either end of the amplified rmFH-coding sequence to allow insertion into pPICZαB upstream of the DNA coding for the alpha factor-secretion signal (vector map Appendix B). Two extra base-pairs (highlighted below) were required to keep

the forward primer sequence in-frame. The first six base-pairs (*italics*) are overhangs due to primer design.

Forward primer (***Pst*II**): 5GAT AAT **CTG CAG** GAG AGG
ATT GTA AAG GTC CAC³

Reverse primer (***Xba*I**): 5GGC GCC **TCT AGA** CTA CTA
AAC ACA AGT TGG GTA GTTG³

The PCR reaction mix consisted of 1 µl mini-prep plasmid DNA (~200 ng), 5 µl of 10x Cloned Pfu buffer (Stratagene), 1 µl of deoxynucleotide triphosphates (dNTPs) (2.5 mM, Invitrogen), 1 µl of each forward and reverse primer (25 µM stock, Invitrogen), 1 µl (2.5 U/µl) Pfu Turbo DNA polymerase (Agilent), 1.5 µl dimethylsulfoxide (DMSO) (Stratagene) and 38.5 µl double-distilled water to make a total reaction volume of 50 µl.

The thermal cycling parameters are shown in Table 2.1 and were performed by an Eppendorf Mastercycler Personal in 0.25-ml thin-walled PCR tubes.

Step	Temperature (°C)	Time (min)	Repetitions
Initiation	95	5	-
Denaturation	95	0.5	35 x
Annealing	68	0.5	
Extension	72	5	
Final extension	72	10	-
Hold	4	Hold	-

Table 2.1 Amplification PCR program. The annealing temperature was set according to the lowest calculated melting temperature (T_m) of the primer pair with 5 °C subtracted.

2.1.1.2 DNA-agarose gel electrophoresis

A 1% w/v agarose solution in 50 ml Tris-acetate-EDTA (TAE) buffer was prepared by heating in a microwave oven until the agarose had completely dissolved. A 5 µl aliquot of SYBR Safe stain (Invitrogen) was added and the mixture was poured into a tank containing a comb with the desired number of wells. Once the gel had set, a 100-bp or 1K-bp DNA size ladder (New England Biolabs, NEB) was loaded alongside 5 µl of the PCR product. The gel was run at 125 V, until clear resolution of the size-ladder bands had occurred. DNA bands were visualized using UV light on a benchtop transilluminator (3UV Transilluminator, UVP). A visible DNA band signified successful DNA amplification.

2.1.1.3 Restriction enzyme digestion

A restriction enzyme double digest of the amplified DNA was performed using *Pst*I and *Xba*I. The digest mixture contained the amplified DNA (45 µl), buffer 3 (5 µl), *Pst*I and *Xba*I (1 µl each of 20,000 units/ml), 1 µl of bovine serum albumin (BSA) (100 mg/ml). All reagents were provided by NEB. Reaction Buffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂ and 1 mM DTT pH 7.9) was used to maximise the efficiency of both restriction enzymes, following NEB recommendations for performing a double digest. The reaction components were mixed and incubated at 37 °C for two hours, then deactivated at 65 °C for 20 minutes.

2.1.1.4 Ethanol precipitation

Ethanol precipitation was performed by adding a quantity, equating to 10% of the total reaction volume, of 3 M sodium acetate, pH 5.2, plus 2.5 x the volume of ice-cold 100% ethanol. The mixture was vortexed and placed on ice, or at -20 °C, for 1 hour. The mixture was then centrifuged at 16,100 g for 30 minutes at 4 °C, and the supernatant removed with a pipette and discarded. The pellet was washed with 100% of the initial reaction volume of ice-cold 70% (v/v) ethanol and centrifuged at 16,100 g at 4 °C for 10 minutes. The supernatant was aspirated, and the tubes containing the pellets were spun for a further 10

minutes to allow any residual ethanol to be removed. The pellet was air-dried, then re-suspended in 10 µl of double-distilled water (ddH₂O).

2.1.1.5 Quantification of DNA

Concentrations of DNA were determined by spectrophotometry using a Biospectrometer (Eppendorf) to measure absorbance at a wavelength of 260 nm. The purity of the DNA was estimated by measuring the ratios of $A_{260\text{ nm}}/A_{280\text{ nm}}$ and $A_{260\text{ nm}}/A_{320\text{ nm}}$.

2.1.1.6 Ligation reaction

Approximately 50 ng of cut pPICZαB vector was combined with 150 ng of insert (*i.e.* the double digested amplified product), and total volume adjusted to 10 µl with ddH₂O. Aliquots of 10 µl of 2x Quick Ligation Reaction Buffer (NEB) and 1 µl (400 units/µl) of Quick T4 DNA ligase (NEB) were added. Following mixing and brief centrifugation at 16,100 g, the mixture was left at room temperature for 300 s before transformation of *E. coli*.

2.1.1.7 Transformation of *E. coli*

A 2 µl aliquot of ligation mixture was added to 50 µl of chemically competent XL1 Blu *E. coli* cells (Agilent). The cells were incubated on ice for 30 minutes, then subjected to heat shock for 45 seconds in a 42 °C water bath, followed by a further 120 s on ice. To this was added 250 µl of pre-warmed SOC media (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose, Invitrogen), and the cells agitated at 37 °C for one hour. The cells were plated onto low-salt LB agar containing 25 µg/ml Zeocin (Invitrogen) and incubated overnight at 37 °C.

2.1.1.8 Screening PCR

Eight colonies from the transformed *E. coli* plate were screened for the correct insert. Each colony was re-streaked onto a fresh low-salt LB agar plate (with 25 µg/ml Zeocin) and incubated at 37 °C overnight. A colony from each re-streaked plate was transferred to a separate PCR tube containing 20 µl of EB buffer (10 mM Tris pH 8.5, QIAprep Miniprep kit) and heated at 95 °C for 300 s to lyse the bacteria and solubilise the plasmid DNA. Of this mixture, 2 µl was

added to 5 µl PCR Mastermix solution (Promega) and 2 µl (25 nM stock supplied by Invitrogen) of each forward and reverse primer (gift from Dr Nicola McIntosh).

Forward primer 5'-3': GGGGATTTTCGATGTTGCTGTT

Reverse primer 5'-3':
CCGGTCTTCTCGTAAGTGCC

Thermal cycling parameters are shown in Table 2.2.

Step	Temperature (°C)	Time (min)	Repetitions
Initiation	95	1	-
Denaturation	95	0.5	39 x
Annealing	55	0.5	
Extension	72	5	
Final extension	72	4	-
Hold	4	Hold	-

Table 2.2 Screening PCR program

Each colony was tested for successful ligation by analysing each PCR product using DNA agarose gel electrophoresis (2.1.1.2). Colonies showing an insert of the correct size (~ 4 kilobase-pairs) were grown overnight at 37 °C in 5 ml low-salt LB medium with 25 µg/ml final concentration Zeocin. Plasmid DNA was extracted using QIAprep Spin Miniprep kit (Qiagen) by following the manufacturer-provided protocols.

2.1.1.9 Sequencing PCR

DNA sequencing was performed by the automated sequencing service 'Genepool' at the University of Edinburgh. Nine forward and reverse primers (Appendix C) were designed to check the entire rmFH sequence, plus look for

evidence of integration into the pPICZαB vector. A 300 ng sample of template DNA, 4 µl of ABI prism BigDye Terminator Mix V3.1 (Applied Biosystems) and 1 µl of primer (10 µM stock) was combined with ddH₂O to make a total reaction volume of 20 µl. Thermal cycling parameters are shown in Table 2.3.

Step	Temperature (°C)	Time (min)	Repetitions
Initiation	95	1	-
Denaturation	96	0.5	30 x
Annealing	50	0.5	
Extension	60	1	
Hold	4	Hold	-

Table 2.3 Sequencing PCR program

2.1.1.10 Large-scale production of DNA

A colony with the correct insert, according to sequencing, was grown in 5 ml of low-salt LB medium (25 µg/ml final concentration Zeocin) at 37 °C for eight hours; this was subsequently used to inoculate 250 ml low-salt LB medium (25 µg/ml Zeocin) and grown at 37 °C overnight (for “maxi-prep”). Plasmid DNA was extracted using the Qiagen HiSpeed Plasmid Purification kit according to manufacturer’s instructions.

2.1.1.11 Linearization of DNA

Plasmids must be linearized prior to transformation into *P. pastoris* cells. This was achieved with *SacI* that cuts the vector but not within its coding regions. A 440 µl sample of plasmid (from the maxi-prep), 6 µl *SacI* (20,000 units/ml), 50 µl Buffer 1 (10 mM Bis-Tris-Propane-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.0) and 5 µl BSA (100 mg/ml) were mixed and incubated at 37 °C for six hours.

An aliquot of the product was run on a DNA agarose gel (2.1.1.2) alongside non-linearized plasmid to check for the expected mobility shift.

2.1.1.12 Phenol-chloroform extraction

To eliminate residual restriction enzymes and yield pure DNA, phenol-chloroform extraction was performed on the linearised plasmid. To a volume of the DNA, an equal amount of phenol:chloroform:isoamyl alcohol (25:24:1, saturated with 10 mM Tris containing 1 mM EDTA, pH 8.0) was added. The tube was inverted several times (until an emulsion formed), then centrifuged at 16,100 g in a bench-top centrifuge (Eppendorf) (4 °C, 60 s). The upper aqueous layer was transferred to a fresh tube and the extraction process repeated. An equal volume of chloroform was added to the aqueous layer before repeating the centrifugation process. Finally, the aqueous layer was removed and subjected to ethanol precipitation (2.1.1.4) of pure DNA.

2.1.2 Recombinant murine factor H in pJexpress912

An alternative expression-optimised FH gene was purchased from DNA 2.0. The gene was supplied within the *P. pastoris* vector pJexpress912 (vector map Appendix B), which (like pPICZα), utilises Zeocin selection and codes for the alpha factor-secretion signal.

The DNA was provided as a maxi-prep sample. For integration of genetic material at the AOX1 locus, the plasmid was linearised. A sample of 100 µg of DNA (10 µg/µl stock), 50 µl *Swa*I (10,000 units/ml), 5 µl BSA (100 mg/ml) and 50 µl buffer 3 (as in 2.1.1.3) was added to 385 µl ddH₂O to make a 500 µl reaction volume, which was incubated at 37 °C for one hour (all reagents were from NEB).

For integration at the AOXI terminator, linearization with *Bam*HI was required. Samples of 100 µg DNA (10 µg/µl stock), 5 µl *Bam*HI (100,000 units/ml) and 50 µl buffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9) were added to 435 µl ddH₂O to make a total reaction volume of 500 µl, which was incubated at 37 °C for one hour.

Subsequent information from the supplier (DNA 2.0) suggested linearization with *SacI* may improve expression. A sample of 100 µg of DNA (10 µg/µl stock), 6 µl *SacI* (20,000 units/ml), 5 µl BSA (100 mg/ml) and 50 µl buffer 1 (as in 2.1.1.11) was added to 429 µl ddH₂O to make a total reaction volume of 500 µl, which was incubated at 37 °C for 5 hours.

Phenol-chloroform extraction and ethanol precipitation were performed prior to transformation into *P. pastoris* as previously described.

2.1.3 Protein Disulphide Isomerase (PDI)

Protein Disulphide Isomerase (PDI) is an endoplasmic reticulum chaperone protein which assists in the reformation of non-native disulphide bonds. An expression-optimised DNA sequence encoding the *P. pastoris* PDI gene (Appendix F for sequence) was purchased in the vector pPIC3.5K (vector map Appendix B) from GeneArt Life Technologies.

Transformation of *E. coli* and maxi-prep, linearization with *SacI*, and phenol-chloroform extraction and ethanol precipitation of DNA were all performed as previously described.

2.1.4 Recombinant human complement factor H (rhFH)

The human FH amino acid sequence contains several common polymorphisms including I62V, Y402H and D936E. V62 and H402 are associated with a significantly increased life-time risk of developing age-related macular degeneration (148-151). E936D is associated with an increased susceptibility to meningococcal disease (152). The original rhFH produced in *P. pastoris* KM71H strain (23) contained V62, Y402 and E936 (V62-rhFH). In designing the rhFH mutants it was decided that the new rhFH root gene should contain I62, Y402 and D936 (I62-rhFH) to reduce potential confounding results in functional assays.

The expression-optimised DNA encoding I62-rhFH and the aHUS-associated mutants D1119G (D1119G-rhFH), R53H (R53H-rhFH) and S1191L V1197A (LA-rhFH) were all obtained from GeneArt. Transformations of *E. coli* and

maxi-prep, linearization with *SacI*, phenol-chloroform extraction and ethanol precipitation were performed as previously described.

2.2 Recombinant protein production in *Pichia pastoris*

2.2.1 Overview

As previously discussed *P. pastoris* is a yeast expression host that was used to produce recombinant human FH (23). As a eukaryotic expression system, *P. pastoris* has important advantages over prokaryotic systems, such as the ability to carry out post-translational modifications, including glycosylation, proteolytic processing and disulfide bond formation (153-155) as well as correct protein folding.

P. pastoris can use methanol as a sole carbon source through a methanol metabolic pathway. The initial step in methanol metabolism occurs when alcohol oxidase (AOX) catalyses the oxidation of methanol to formaldehyde creating hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism is localised to the peroxisome, an organelle that sequesters toxic by-products. Alcohol oxidase has low affinity for O₂ so yeast cells compensate by producing copious enzyme. The promoters concerned are, consequently, exceptionally strong; and they are frequently exploited to obtain high yields of inducible recombinant proteins (155).

P. pastoris has two genes encoding AOX, *AOX1* and *AOX2*. Whilst *AOX2* is 97 % similar to *AOX1*, growth that relies on methanol, and hence the activity of alcohol oxidase, is much faster when *AOX1* is responsible (156). Thus, when methanol is provided as the carbon source for growth of *P. pastoris* cells, *AOX1* - or any recombinant gene under control of the *AOX1* promoter - is expressed at very high levels that are regulated at the level of transcription. Regulation relies both on repression/derepression and induction. Importantly, glucose represses transcription even if methanol (the inducer) is present. Hence, cells used for recombinant gene expression are grown up to a high

density in glucose prior to induction of *AOX1*-dependent genes by methanol, whereupon glucose is exchanged for glycerol for a protein-production phase.

P. pastoris has two methanol-metabolizing phenotypes: Mut^S and Mut⁺. The Mut^S phenotype (methanol utilization slow) results from the loss of the *AOX1* gene and hence the majority of alcohol oxidase activity. Hence this strain relies on *AOX2* and grows on methanol slowly. The strain with phenotype Mut⁺ (methanol utilization plus) refers to the wild-type ability of strains to grow on methanol as a sole carbon source. The Mut^S *P. pastoris* strains used for the majority of work in this project were KM71H and KM71.

In the *P. pastoris* expression system, heterologous proteins can either be expressed intracellularly, or secreted by using a signal sequence to target a protein to the secretory pathway. Various secretion signals have been used for recombinant protein production, including a *P. pastoris* native secretion signal. Most success, however, derived from utilizing a secretion-signal sequence from the yeast *Saccharomyces cerevisiae*, the prepro alpha-mating factor (153). Note that *P. pastoris* secretes low levels of its native proteins. This, combined with use of simple media for growth, results in the recombinant target dominating protein in the media, thus facilitating purification.

Utilizing the strongly inducible *AOX1* promoter for expression, the plasmid pPICZαB (Invitrogen) was previously used as a vector in the production of rhFH (23). The plasmid contains a version of the *AOX1* promoter which is used to drive expression of the heterologous gene of interest. The plasmid also carries DNA encoding the alpha-factor secretion signal peptide. This is fused to the inserted gene to ensure secretion of the target protein. Using this vector thus ensures that once the heterologous protein-encoding sequence has been integrated into the genome of *P. pastoris*, the *AOX1* promoter is responsible for expression and this can be modulated by feeding with methanol.

The N-terminus of the expressed protein may include one or two non-native Glu-Ala repeats due to processing. Two steps are required for processing the alpha-factor signal sequence. The first involves cleavage by the *KEX2* gene

product between Arg and Glu in the sequence: Glu-Lys-Arg* Glu-Ala-Glu-Ala (* is the site of cleavage) (157). The second step involves cleavage of the Glu-Ala repeats by the *STE13* gene product. *Ste13* cleavage may be inefficient, leaving non-native N-terminal Glu-Ala or Glu-Ala-Glu-Ala (158).

2.2.2 *P. pastoris* strains

KM71H (Mut^S), X33 (Mut⁺) and SMD1168H (Mut⁺, Protease A deficient) were obtained from Invitrogen. KM71 (Mut^S) was obtained from LGC Standards (Middlesex, U.K.). Note that KM71 is auxotrophic for histidine.

2.2.3 Preparation of electrocompetent cells

A 5-ml aliquot of yeast-peptone-dextrose (YPD) medium was inoculated with a single *P. pastoris* colony and grown overnight at 30 °C in a shaking incubator. Three 100-ml cultures containing fresh YPD medium were inoculated with 20, 40 or 80 µl of the 5-ml culture and grown overnight at 30 °C. The culture reaching an OD₆₀₀ closest to the required value of 1.3-1.5 was selected for harvesting by centrifugation (1500 g for 300 s) at 4 °C. After re-suspending the pellet in 100 ml ice-cold sterile water it was spun again and the pellet re-suspended in 50 ml ice-cold sterile water. After a further spin, the pellet was re-suspended in 4 ml of ice-cold sterile 1 M sorbitol. Following a final centrifugation step, the pellet was re-suspended in 200 µl ice-cold 1 M sorbitol. Cells were stored on ice and used on the day they were prepared.

2.2.4 Transformation of *P. pastoris* by electroporation

An 80-µl aliquot of these electrocompetent cells was added to linearised plasmid (~ 30 µg in maximum 10 µl sterile water) then transferred to an ice-cold electroporation cuvette (0.2-cm Genepulser, Biorad). The cuvette was incubated on ice for 300 s then pulsed at 1.5 V for 6 s (Biorad Genepulser II). Immediately, 1 ml of either ice-cold 1 M sorbitol or yeast-peptone-dextrose-sorbitol (YPDS) medium was added to the cuvette. The cuvette contents were transferred to a pre-chilled 15-ml tube and incubated without shaking at 30 °C for three hours. Aliquots of 200 µl were then spread on YPDS plates, with a

final Zeocin concentration ranging from 100 µg/ml to 500 µg/ml. Plates were incubated at 30 °C until colonies had grown (three-five days).

Transformed colonies were re-streaked onto fresh YPDS agar plates (containing the same Zeocin concentration as the original plate) then incubated for three days at 30 °C.

2.2.5 Small-scale flask expression tests

2.2.5.1 KM71H

Test expressions were performed in shaker flasks to identify the best protein-producing clone(s) for use in subsequent fermentation. Plasmids pPICZαB containing DNA for rmFH or rhFH, and pJe912 containing DNA for rmFH, were transformed into KM71H.

Single colonies were used to inoculate 5 ml of buffered glycerol-complex medium (BMGY) in a 50-ml Falcon tube and grown at 30 °C in a shaking incubator for 24 hours. Multiple colonies were tested in parallel. Each 5-ml culture was added to 100 ml of BMGY in a 500-ml baffled flask and grown at 30 °C in a shaking incubator for 48 hours. Cells were harvested by centrifugation (3000 g, 15 minutes) at room temperature, and the pellet re-suspended in 25 ml buffered methanol-complex medium (BMMY). The suspension was returned to the baffled flask for further growth in the shaking incubator (room temperature). At 24-hour intervals, methanol was added to a final concentration (v/v) of 0.5% - 1%. On the fourth day, supernatant was harvested by centrifuging (3000 g, 15 minutes) and the cell pellet discarded. The supernatant was filtered using a 0.22-µm sterile filter (Millipore) and the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and calcium-chelator ethylenediaminetetraacetic (EDTA) were added to give final concentrations of 0.5 mM and 5 mM respectively. Protein production was checked using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie staining or Western blotting (WB).

2.2.5.1.1 Heparin-affinity purification of rmFH (pJe912 in KM71H)

A heparin-affinity purification step was used for test expressions, in KM71H *P. pastoris*, of the codon-optimised rmFH gene (DNA 2.0) in the pJe912 vector. A 1-ml HiTrap Heparin (GE Healthcare) column was washed with 10 ml of elution buffer (20 mM potassium phosphate, pH 6, 1 M NaCl). The column was then equilibrated with 10 ml of binding buffer (20 mM potassium phosphate, pH 6, 100 mM NaCl). The supernatant from the KM71H cell culture was diluted 1:3, then syringed onto the column. The column was washed with 2 ml of binding buffer, before protein elution using five 300- μ l aliquots of elution buffer with fractions collected separately. An aliquot from each fraction was deglycosylated (see below 2.4.9) and run on SDS PAGE, and then protein was detected by SDS-PAGE and WB.

2.2.5.2 X33 and SMD1168H

The plasmid pJe912 containing the rmFH gene was also transformed into X33 (Mut⁺) and SMD1168H (Mut⁺, Protease A deficient) strains using the procedures described above. For expression tests, the 5-ml inoculum (prepared as above) was added to 25 ml of BMGY and incubated at 30 °C in a shaking incubator for 24 hours. The cell pellet was harvested by centrifugation, then re-suspended into 100 ml BMMY in a 1-l baffled flask. The culture was methanol-fed and harvested in the same way as KM71H.

2.2.5.3 KM71 and PDI

Vector pPIC3.5K containing the *PDI* gene was transformed into KM71 cells. This strain has a mutation in the *HIS4* gene (*HIS4*⁻). To grow in media lacking histidine, it requires plasmids containing *HIS4*, such as pPIC3.5K, and only successfully transformed clones will grow in minimal media. Hence cells were plated on minimal dextrose (MD), incubated (30 °C) for three-five days, then re-streaked on MD plates for three days of further growth. Test expressions (of *PDI* from KM71) were then performed using the procedure for test expressions of rmFH in KM71H described above. As PDI does not have a secretion signal, both supernatant and cell pellet were retained post-harvest for testing.

To obtain protein from the pellet, 500 µl of 4x NuPAGE LDS sample buffer (106 mM Tris HCl, 141 mM Tris Base, 2% w/v lithium dodecyl sulfate, 10% v/v glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM phenol red, pH 8.5, Invitrogen) was added to 500 µl of cells taken from the pellet and vortexed. The mixture was incubated at 100 °C for 120 s in a heat block then re-vortexed. This process was repeated four times and then the mixture was centrifuged (600 s, 16,100 g). The resulting supernatant was tested by SDS-PAGE for protein expression.

The resultant *PDI*-expressing strain of KM71 was transformed with plasmids containing the DNA encoding rmFH. Subsequent test expressions were performed as for KM71H.

2.2.6 Five-litre fermentation

2.2.6.1 Standard protocol

Growth in a fermentor achieves high cell densities thereby increasing yields of recombinant protein. Fermentations were performed on a scale of five litres in a BioFlo 3000 (New Brunswick Scientific), which allows temperature, agitation, pH and air flow to be adjusted. Initial settings followed Invitrogen guidelines (https://tools.thermofisher.com/content/sfs/manuals/pichiaferm_prot.pdf).

A single colony of genetically modified *P. pastoris* cells was re-streaked on a YPDS (yeast-peptone-dextrose-sorbitol) plate in the presence of Zeocin, then incubated at 30 °C for three-five days. A 10-ml starter culture was prepared by inoculating YPD (yeast extract plus peptone and dextrose) medium with a single colony and incubating at 30 °C for two days in the presence of Zeocin. This was used to inoculate 500 ml of BMG (buffered minimal glycerol) plus 100 ml YPD (total volume 600 ml) and incubated (shaking incubator) at 30 °C for a further two days. The starter cultures contained varying concentrations of Zeocin as discussed in the relevant results section.

Whilst these cultures were incubating, the fermentor was prepared. The basal salts media (Table 2.4) was prepared in a total volume of 2.5 L ddH₂O and added to the vessel which was then autoclaved.

Phosphoric acid	27 ml/L
Calcium sulphate	0.95 g/L
Potassium sulphate	18.2 g/L
Magnesium sulphate heptahydrate	15 g/L
Potassium hydroxide	4.2 g/L
Glycerol	25 ml/L
Water	Up to 2.5 L

Table 2.4 Basal salts medium for standard 5-L fermentation

After autoclaving the fermentor vessel, the dissolved oxygen (DO) probe was attached and charged overnight by setting the agitation speed to 200 rpm (and temperature to 30 °C) and the air flow to “on” so that the medium became saturated with air. Next morning the DO probe was calibrated, the base feed-line supplying 34% (v/v) ammonia solution (Sigma Aldrich) was attached and an agitation/DO “cascade” was set-up; the agitation speed was set (over a range of 50 rpm to 1000 rpm) to maintain the DO at 40% (of the reading at saturation) during the period of growth and induction. Hence, the agitation speed reflects the rate of metabolism (oxygen consumption) by the cultured cells and can be used to monitor the utilisation of the carbon source.

Following addition of the starter culture, the pH was adjusted to 5.0 using a few ml of ammonium hydroxide and the fermentation (at 30 °C) was initiated with an initial agitation speed of 200 rpm. Cell mass initially increased due to growth that was sustained using a manual, batch-mode glycerol feed (approx. 100-200 ml every four-eight hours) for 24-36 hours. Feeding was modulated according to utilisation as judged by oxygen consumption/agitation speed.

Prior to methanol induction of recombinant FH production the temperature of the fermentor was stabilised at 15 °C. Methanol feeds (batch mode, 0.5 - 1.5 % of culture volume) over the next three days were modulated according to consumption judged by agitation speed. Both glycerol and methanol feeds contained PTM₁ Trace Salts (4.35ml/L Amresco). Aliquots of 0.5-ml Anti-Foam 204 (Sigma Aldrich) were added as needed.

2.2.6.2 Low-salt protocol

The basal salts media was altered to provide an alternative low-salt base for the fermentation (Table 2.5).

Phosphoric acid	16 ml/L
Calcium sulphate	0.64 g/L
Potassium sulphate	4.55 g/L
Magnesium sulphate heptahydrate	2.5 g/L
Potassium hydroxide	0.625 g/L
Glycerol	28 ml/L
Water	Up to 2.5 L

Table 2.5 Basal salts medium low salt 5-L fermentation

The standard feeding regime described in the previous section was implemented. In addition, at 24-hour intervals following methanol induction, 1 % w/v casamino acids (Millipore) were added.

In a second low-salt fermentation, the PTM₁ salts in the methanol feed were replaced with 10 mM PMSF while PTM₁ salts were added separately.

2.2.7 Ten-litre fermentation

2.2.7.1 Standard protocol

Ten-litre fermentations were performed by Dr John White (School of Chemistry) using a BioFlo 4500 (New Brunswick Scientific) fermentor and a protocol that had previously proved successful for rhFH. Two 500-ml BMGY cultures (with varying Zeocin concentrations) were grown for 48 hours at 30 °C prior to inoculation into the fermentor. The basal salts recipe was the same as used for standard five-litre fermentation, adjusted for a 7.5 L initial volume. Glycerol feeds were performed as described above. Post-induction, batches of 150 ml methanol/PTM₁ salts, containing also 1% sorbitol (w/v) and 0.3% tryptone (w/v), were added to the fermentor manually according to consumption as judged by agitation speed.

Initial ten-litre fermentations for production in KM71H of rmFH and D1119G-rhFH were performed using this method. A four-day methanol-induction phase was used for ten-litre fermentations of V62-rhFH (in KM71H), and rmFH (in X33). Note that X33 cells received 200-ml, rather than 150-ml, methanol/PTM₁ salts feeds.

2.2.7.2 Low-pH protocol

For growths at low pH, the initial set-up of the fermentor and preparation of the basal salts medium was the same as used in the standard ten-litre fermentation. Between the third and fourth glycerol feeds, the pH was adjusted to 3.0 (from 5.0) and the culture allowed to acidify prior to induction with methanol. Methanol induction was then performed using the same procedures as used in the standard fermentations (as described above).

2.2.7.3 Batch feeding versus continuous (pump) feeding

The potential benefits of using a peristaltic pump for feeding cells rather than providing batches of feed manually were explored, along with the effects of replacing feeds of methanol with a 4:1 mixture of methanol and glycerol. In total, four ten-litre scale fermentations for production of rmFH in KM71 PDI cells were set up as before, with the starter culture grown in YPG. In the first two fermentations – fermentation 1 batch-fed and fermentation 2 pump-fed

during a four day induction phase - PMSF was dissolved in methanol/glycerol feeds (10 mM, *i.e.* 3.48 g per 2 L bottle) while PTM₁ salts (12 ml per L of feed) were added separately. For the pump-fed fermentation (2), a peristaltic pump was set to continuously feed methanol/glycerol at an initial rate of 50 ml/hour, which increased each day to reach 88 ml/hour by the fourth day following initial induction. In both fermentations 1 and 2, additions to achieve a final concentration of 0.3% tryptone (w/v) were made every four hours, while the 1% sorbitol was omitted.

A third and a fourth ten-litre scale fermentation both used continuous (pump) feeding of methanol/glycerol and a four-day induction phase. In fermentation 3, 250 mg of Pefabloc SC (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, AEBSF, Sigma Aldrich), a protease inhibitor, was added every 24 hours during the induction phase, while in fermentation 4, anhydrous methanol/glycerol provided during induction contained 10 mM PMSF (anhydrous). Tryptone and PTM₁ salts were supplied as previously described. Fermentation 3 protocol was also used to produce I62-rhFH and the mutants R53H-rhFH and LA-rhFH.

2.2.8 Harvesting

For harvesting, cultures were removed from the fermentor and centrifuged at 5000 g for 15 minutes at 4 °C (centrifuge: Sorvall RC3BP⁺, Thermo; rotor: Sorvall H-6000). The supernatant (containing secreted FH) was transferred to fresh bottles and re-centrifuged at 8000 g for 30 minutes and 4 °C (centrifuge: Sorvall^R RC6PLUS; rotor: FIBERlite F13-14x50cy, Thermo). Then cell-free supernatant was decanted, and EDTA and PMSF added to final concentrations of 5 mM and 0.5 mM, respectively.

The supernatant was diluted approximately 1:6, using distilled water, to achieve a conductivity equivalent to that of 50-75 mM sodium chloride (NaCl), in preparation for ion-exchange chromatography. Finally, additional EDTA, to a final concentration of 5 mM, was added to the diluted supernatant.

2.3 Detection of Factor H

2.3.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

All reagents were purchased from Invitrogen other than the protein marker ladder (BioRad). Precast NuPAGE Bis-Tris gradient (4-12% agarose) gels (Invitrogen) were used. Sample volumes varied between 10 and 25 μ l depending on the number of sample wells (10, 12, 15 or 18). Samples for SDS-PAGE were prepared by mixing the test sample with NuPAGE LDS Sample Buffer (4X). If reduced samples were required, a 1/10th volume of NuPAGE Sample Reducing Agent (10X) was added (contains 500 mM dithiothreitol). The samples were then heated at 100 °C for 120 s and briefly centrifuged (at 16,100 g) prior to loading on the gel. The gels were run in NuPAGE MOPS (Invitrogen) buffer at 200 V for one hour. Precision Plus (BioRad) protein marker (10-250 kDa range) was run alongside the samples. Protein bands were visualised by staining gels with Bio-Safe Coomassie stain (BioRad), or in-house laboratory-made Coomassie stain, or by silver staining (see below).

2.3.1 Silver staining

Silver staining provides greater sensitivity than Coomassie Blue staining. For silver staining, gels were soaked for 600 s in fixing solution (40% v/v methanol and 13.5% v/v formalin in H₂O). They were then washed in H₂O and soaked for 60 s in 0.02% w/v sodium thiosulphate solution prior to a further wash with H₂O. Gels were then soaked for 600 s in 0.1% w/v aqueous silver nitrate, prior to rinsing with H₂O and a small volume of developing solution (3% w/v sodium carbonate, 0.05% v/v formalin, 0.000016% w/v sodium thiosulphate in H₂O). Next, the gel was soaked in fresh developing solution (as above) until bands became visible (60-300 s). Subsequently it was washed for 600 s with 12.5 ml 2.3 M citric acid, followed by several washes with H₂O.

2.3.2 Western blot

Western blots (WBs) were used to detect both plasma-derived and rFH. Samples for WB were run on a NuPAGE gel, but instead of staining, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad)

using an XCell II™ Blot Module (Invitrogen) at 30 V for one hour in 1x transfer buffer (Invitrogen). After transfer the membrane was placed in a 50-ml Falcon tube and incubated, at 4 °C for two hours on a roller, with 40 ml blocking buffer (PBS with v/v 5% milk powder) or SuperBlock Blocking Buffer (ThermoFisher Scientific). The membrane was then incubated overnight at 4 °C on a roller with primary antibody (Table 2.6) in 10 ml of blocking buffer.

Next day the membrane was washed in 10 ml PBS for 20 minutes, subjected to two 20-minute washes with 10 ml PBS containing 0.05% v/v Tween 20 (Biorad), then a further two 20-minute washes with PBS. The membrane was then incubated (4 °C, two hours) on a roller with secondary antibody (Table 2.6) dissolved in 5 ml blocking buffer. The membrane was then washed in 10 ml PBS for 300 s, followed by two 600-s washes with 20 ml PBS containing 0.05% v/v Tween 20, and a final 300-s wash with PBS. Protein was visualised using SIGMAFAST™ 3,3-Diaminobenzidine tablets.

Table 2.6 shows the primary antibodies and relevant secondary antibodies used for mFH detection at various stages in FH production/purification. See Appendix D for assessment of antibodies in rmFH detection.

Primary Antibody	Secondary Antibody
Rabbit antibody to mouse FH 1:200 (Santa Cruz)	Goat antibody to rabbit IgG 1:1000 (Cell Signalling)
Goat antibody to human FH 1:200 (Calbiochem)	Rabbit antibody to goat IgG 1:2500 (Sigma)
Sheep antibody to human FH 1:200 (Abcam)	Rabbit antibody to sheep IgG 1:20,000 (Abcam)

Table 2.6 Western blot antibodies

2.4 Factor H purification from *Pichia pastoris* culture

Protein purification protocols were developed from a previously published method for rhFH purification (23). All chromatography steps (unless stated otherwise) were carried out using an ÄKTA FPLC system (GE Healthcare) employing columns and chromatography media supplied by GE Healthcare. Volumes of protein-containing solution involved at each stage in different fermentations are discussed in relevant results section.

2.4.1 Cation-exchange chromatography

As an initial purification step for FH, an XK 26/20 column was packed with 75 ml of SP Sepharose Fast Flow resin. The column was first equilibrated in binding buffer (20 mM potassium phosphate, pH 6, 50 mM NaCl (100 mM NaCl in fermentation one)). After applying dilute supernatant to the column, it was washed with two column volumes (CV) of binding buffer, then eluted over four or five CV with a gradient from 100% binding buffer to 100% elution buffer (20 mM potassium phosphate, pH 6, 1 M NaCl) at a flow rate of 5 ml/min.

The initial cation-exchange step for rmFH produced in PDI -KM71 cells used a XK 26/20 column packed with 50 ml of Toyopearl SP-650 resin (Tosoh Biosciences). The binding buffer was 20 mM potassium phosphate, pH 6, 50 mM NaCl, and the elution buffer contained 20 mM potassium phosphate, pH 6 and 0.5 M NaCl.

Fractions containing protein, as identified using SDS-PAGE with Coomassie staining, were pooled and subjected to de-glycosylation by Endo H_r (NEB) (section 2.4.9).

2.4.2 Lectin-affinity chromatography

Concanavalin A (Con A) is a lectin (carbohydrate-binding protein) used for the purification of glycoproteins. In this instance it was used to remove the *P. pastoris*-derived N-glycans that had been cleaved by Endo H_r and were still present in the protein solution.

A bench-top column was packed with 5 ml of Con A resin and this was equilibrated in two CV of 20 mM potassium phosphate, pH 6, 200 mM NaCl (binding buffer). The protein solution (see individual fermentations) was applied manually to the column and the flow-through (non-glycosylated species) was collected. The column was further washed with two 10-ml aliquots of binding buffer to complete elution of the deglycosylated material. Finally two 10-ml aliquots of elution buffer (20 mM potassium phosphate, pH 6, 200 mM NaCl, 1 M methyl α -D-glucopyranoside) were applied to the column to recover any protein that has not been fully de-glycosylated.

Fractions containing de-glycosylated protein (identified using SDS-PAGE, Coomassie staining) were buffer-exchanged prior to anion exchange (see below), using Snakeskin dialysis tubing with a 3.5-kDa molecular weight cut-off (MWCO) (ThermoFisher Scientific). Dialysis bag(s) (volume 50-70 ml) were placed in 4 L 20 mM glycine buffer, pH 9.5 plus 90 mM NaCl. Dialysis proceeded overnight, then the bag transferred to 4 L fresh buffer for a further two hours after which the bag's contents were recovered. Note that rmFH was dialysed at room temperature while rhFH was dialysed at 4 °C.

2.4.3 Anion-exchange chromatography

In an additional purification step, either a Tricorn Mono Q 4.6/100 column (with a 1.7-ml CV) or a self-poured Source 15Q (with a 7.8-ml CV) column was used. Binding buffer in both cases was 20 mM glycine, pH 9.5, 90 mM NaCl while the elution buffer was 20 mM, glycine pH 9.5, 1 M NaCl. Protein was eluted over 25 CV initially, with gradient adjustments made for subsequent runs, once the elution point was known for an individual protein.

Fractions containing the desired protein were pooled and dialysed using Snakeskin membranes into PBS (phosphate buffered saline, pH 7.4) for either immediate freezing and storage at -80 °C, or in preparation for a final “polishing” step.

2.4.4 Size-exclusion chromatography

Size-exclusion chromatography was performed on a Superdex 200 10/300 GL (flow rate 0.5 ml/min, fraction size 0.5 ml/min) or a Superdex 200 HiLoad 26/600 (flow rate 2 ml/min, fraction size 2 ml). The running buffer was PBS.

2.4.5 Ceramic hydroxyapatite chromatography

Reports in older literature suggest ceramic hydroxyapatite (CHA) chromatography can be used to purify human FH from plasma. Notably, CHA removes lower-MWt contaminants not removed by ion exchange (159). Hydroxyapatite is a form of calcium phosphate, and CHA chromatography purifies using a mixed-mode affinity process. Solute species predominantly interact through cation-exchange *via* the negatively charged phosphate groups and/or a metal-affinity effect *via* the calcium atoms (CHA Instruction Manual, Bio-Rad).

Although different contaminants are present in plasma preps compared to protein made in yeast, CHA chromatography was trialled for rmFH purification in the hope of resolving lower-molecular weight contaminants not removed thus far. This trial used Source-Q fractions that had not been pooled (due to being less pure than fractions from the centre of the peak).

In CHA chromatography, proteins can be eluted either by an increasing salt gradient or an increasing phosphate gradient. A self-poured column (BioRad CHA Type II resin, 7.8-ml CV) was used. Protein was dialysed into a binding buffer containing 10 mM sodium phosphate pH, 6.5 and 200 mM NaCl. Protein was subsequently eluted with either 10 mM sodium phosphate, pH 6.5, 2 M NaCl; or 500 mM sodium phosphate pH 6.5, 200 mM NaCl. A final wash with 500 mM NaCl, pH 6.5, was used to remove residual protein bound to the resin. This purified protein is denoted as rmFH-CHA.

2.4.6 Purification using PspCN

This unpublished affinity-chromatography method was developed, and reagents produced, by Dr Andrew Herbert (School of Chemistry). It was used as an initial purification step for the rhFH mutants LA-rhFH and R53H-rhFH.

Other versions of rhFH were not purified in this way as the method had not been developed at the time of their production.

PspCN is a bacterial protein domain which binds tightly and specifically to human FH, and was produced and purified as described previously (160). PspCN was coupled via a coupling buffer (50 mM Tris, 5mM EDTA-Na, pH 8.5) to UltraLink Iodocetyl Gel (Thermo Scientific) to create the PspCN-affinity resin. It had been established that 1.0 ml of resin could bind 2 mg of FH. Supernatant from a ten-litre *P. pastoris* fermentation was harvested as described above. A batch of 8 ml of the PspCN-affinity resin was mixed with 1 L of supernatant before pouring the slurry into a 20 ml bench-top column (Bio-Rad Econo-Pac polypropylene column). The resin was allowed to settle and then washed with 100 ml HEPES-buffered saline (HBS) containing 0.05% w/v Tween 20 (BioRad). The column was further washed with 100 ml HBS, and then with 100 ml 4 M NaCl to remove non-specific binders (FH remains firmly bound to PspCN under high-salt conditions). The resin was re-suspended between each washing step. Finally, FH was eluted with 100 mM glycine pH 2.5, that was added slowly to the column, using A_{280} measurements of the collected fractions to monitor protein elution. As soon as the A_{280} started to rise, fresh tubes were used to collect further protein-containing fractions. To the eluent, a 1/10th volume of 1 M Tris pH 8.5 was added to neutralise the pH. The fractions judged, from SDS-PAGE, to contain the target protein were de-glycosylated with Endo H_r (see 2.4.9) and further purified by lectin-affinity and anion-exchange chromatography, as described above.

PspCN cannot be used to purify rmFH as it does not bind to murine FH (personal communication, Dr Andrew Herbert).

2.4.7 Concentration of samples

To concentrate protein (e.g. between purification steps or for loading on SDS-PAGE), 2-ml, 6-ml or 20-ml Vivaspin concentrators (Sartorius) with 30 kDa MWCO were used. Concentrators were washed with ddH₂O prior to use to remove glycerol storage solution. They were centrifuged (4,000 g) until the

desired concentration had been achieved at room temperature (4 °C was not used due to concerns that rFH might precipitate at lower temperatures; rmFH could not be concentrated above 1.5 mg/ml without precipitation when deglycosylated).

2.4.8 Estimation of protein concentration

Approximate protein concentrations were determined by UV spectroscopy (Eppendorf BioSpectrometer) at a wavelength of 280 nm. Using the online ProtParam tool from ExPASy (<http://web.expasy.org/protparam>) the extinction coefficient for each protein was estimated based on amino acid composition as deduced from primary sequences. Concentrations were then calculated by applying the Beer-Lambert equation. Sample purity was assessed by the ratios of $A_{280\text{ nm}}/A_{260\text{ nm}}$ and of $A_{280\text{ nm}}/A_{320\text{ nm}}$.

2.4.9 Enzymatic deglycosylation of N-linked glycans

All rFH samples required treatment to remove *P. pastoris*-derived N-linked glycans. This was important for purposes of SDS-PAGE-based detection and estimation of rFH yield (since glycosylated material runs as a smeary band) in small-scale production trials as well as in full-scale production runs when it was performed after cation-exchange or PspCN-affinity harvesting, and prior to further purification and characterisation steps. Endo H_r (a recombinant fusion of endoglycosidase H and maltose binding protein) cuts between the first two GlcNAc molecules of the mannose-rich N-linked carbohydrate residues, leaving the protein with one residual GlcNAc.

For test productions, 50 µl of supernatant was treated with 1 µl of a stock 1:3 dilution (with ddH₂O) of Endo H_r (1,000,000 units/ml, NEB) for one hour at 37 °C prior to SDS-PAGE. For large-scale fermentation samples, 3 µl of undiluted Endo H_r was added for every 1 ml of eluted protein solution. PMSF (0.5 mM) was also included in the incubation (at 37 °C for one hour; large-scale samples only), prior to preparation of the sample for anion exchange. Note that Endo H_r appears on SDS PAGE as a band at ~70 kDa.

2.5 Factor H purification from mouse serum

This method was adapted from Paixão-Cavalcante *et al* (138). Mouse serum was purchased from Innovative Research (C57BL/6 background) in 100-ml aliquots. A solution of 21% w/v polyethylene glycol (PEG) 8000 (Sigma) was mixed with 25 ml of mouse serum to obtain a final concentration of 7% PEG 8000. This mixture was incubated at 4 °C on a roller for one hour then centrifuged at 10,000 g for 30 minutes. The resultant pellet was dissolved in PBS (~10 ml) and dialysed into 4 L of 20 mM Tris, 50 mM NaCl, pH 7.4 using Snakeskin dialysis tubing (3000 Da MWCO) (ThermoFisher Scientific). Dialysis was performed for four hours at 4 °C, and then overnight using fresh buffer.

Post-dialysis the mixture was filtered using a 0.2-µm sterile syringe filter (Millipore). Two 5-ml HiTrap Heparin HP columns (GE Healthcare) were connected in tandem for an initial affinity purification step. Binding buffer contained 20 mM Tris, 50 mM NaCl, pH 7.4, and the elution buffer contained 500 mM NaCl in place of 50 mM NaCl. The column was extensively washed with binding buffer (25-30 CV) prior to elution. A step-gradient elution was performed, with elution occurring over ten CV to 250 mM NaCl, then a steep gradient to 500 mM NaCl. Fractions were run on SDS-PAGE to check for the presence of mFH, and confirmed by WB.

Mouse FH-containing fractions were dialysed overnight into 4 L of 20 mM glycine, 90 mM NaCl, pH 9.5, then applied to a Source Q column (Section 2.4.3). Fractions were run on SDS-PAGE and mFH bands confirmed by WB.

2.6 Characterisation of recombinant proteins

2.6.1 Mass spectroscopy

2.6.1.1 In-gel trypsin digest

Recombinant murine FH was submitted to the Scottish Instrumentation and Resource Centre for Advanced Mass Spectrometry (SIRCAMS) at the University of Edinburgh for identification of peptide fragments following trypsin digestion.

In preparation for trypsin digestion, the protein sample was run on a 4-12% gradient SDS-PAGE gel, which was subsequently washed three times in 100 ml of water. The gel was stained with InstantBlue protein stain (Expedeon) and washed to remove excess stain. The stained protein band was cut from the gel with the minimal amount of excess polyacrylamide and placed in a 1.5-ml Eppendorf tube. The excised gel segment was washed three times with 300 μ l of 200 mM ammonium bicarbonate (ABC) in 50% v/v acetonitrile (ACN) in water, at room temperature for 30 minutes, to remove SDS. It was then incubated in 300 μ l of 20 mM DTT, 200 mM ABC in 50% ACN for one hour at room temperature to reduce disulfides. The gel segment was finally washed three times in 300 μ l of 200 mM ABC in 50% ACN.

To alkylate cysteine residues, each gel segment was incubated in 100 μ l 50 mM iodoacetamide, 200 mM ABC and 50% ACN, at room temperature, in the dark for 20 minutes. It was then washed thrice with 500 μ l of 20 mM ABC, 50% ACN before being covered with 100 μ l of 100% ACN. Once the gel segment had turned white, ACN was removed and it was dried (37 $^{\circ}$ C for 600 s). The gel segment was then swollen in 29 μ l of 50 mM ABC containing 1 μ l of trypsin stock solution (Promega) (4 $^{\circ}$ C for one hour). The Eppendorf tube was sealed and incubated at 32 $^{\circ}$ C in a heating block for 16-24 hours. Subsequently, the tube was sonicated (in a water bath) for 600 s to extract the digested protein. Data were collected by SIRCAMS using electrospray ionization-mass spectroscopy.

2.6.1.2 Mass of the intact protein

An estimation of the molecular weight of the intact rmFH was based on data collected at the Biomedical Sciences Research Complex Mass Spectrometry Facility, University of St. Andrews.

The protein sample (3 μ L, 1 μ M) was desalted in-line using a nanoLC Ultra 2D plus loading pump and nanoLC as-2 autosampler equipped with a nanoflex cHiPLC chip based chromatography system (Eskigent) and a ChromXP C4-CL trap and column (Eskigent). The protein was eluted with a gradient of

increasing acetonitrile, containing 0.1 % v/v formic acid (5-40% acetonitrile over 300 s, 40-95% over a further 60 s, followed by 95% acetonitrile to clean the column, before re-equilibration to 5% acetonitrile). The eluent was sprayed into a TripleTOF 5600 electrospray tandem mass spectrometer (ABSciex, Foster City, CA) and analysed in “Intact Protein Mode”. The mass spectrum obtained was deconvoluted to give the molecular masses of the main species, using MaxEnt 1 Software (Waters).

2.6.2 Dynamic light scattering

Dynamic light scattering (DLS) was performed at the Edinburgh Protein Production Facility (EPPF). Measurements were performed and analyzed on a Zetasizer Auto Plate Sampler (Malvern). Protein concentrations were ~ 1 mg/ml in PBS. Samples were sterile-filtered (0.2 µm, Millipore) prior to use, then 100 µl of each sample was loaded into a 384-well plate (ThermoFisher Scientific). Each protein sample had three replicates and was assessed by plotting both intensity and volume *versus* apparent hydrodynamic radius.

2.6.3 N-terminal sequencing

Amino-terminal sequencing of rmFH was performed by the MRC Protein Phosphorylation Unit at the University of Dundee.

To prepare the protein, an aliquot of 20 µl, taken from a 1 mg/ml sample, was run on SDS-PAGE then transferred to a PVDF membrane as if for WB (Section 2.3.3). The membrane was stained with Coomassie R-250 (0.1% w/v in 50% v/v aqueous methanol/1% v/v acetic acid) for 60 s and then de-stained with 40% aqueous methanol, 10% v/v acetic acid. The membrane was washed extensively with ddH₂O, then air-dried at room temperature. The required band was excised and placed in an Eppendorf tube for transfer to Dundee.

2.6.4 Comparison of murine and human factor H

Dr Dinesh Soares (MRC Human Genetics Unit, University of Edinburgh) compared human and murine FH CCP 15 sequences (since N-terminal sequencing had demonstrated that clipping occurs within this domain) in order to build a homology model of mFH CCP 15 on the known structure of hFH CCP

15. He used the MODELLER programme (University of California) for which users provide an alignment of the proteins of known and unknown structures, and the programme automatically calculates a 3D structural model containing all non-hydrogen atoms.

2.6.5 Co-factor assay

A fluid-phase co-factor assay was used to measure rmFH co-factor activity on human Factor I (FI)-mediated cleavage of human C3b. The amounts of components used are based on previously published methods (23, 80). Human FH (from serum), FI and C3b were purchased from Complement Technologies (CompTech).

The assay was performed in a final reaction volume of 20 µl in an Eppendorf tube. In each reaction, 850 nM C3b and either 110 nM or 220 nM FI were included. Human FH was injected at 300 nM, while rmFH was applied at both 300 nM and 600 nM. The final reaction volume of 20 µl was attained by addition of PBS. Negative controls contained the same amount of FI and C3b, but lacked FH. The components (without FH) were mixed and incubated in a water bath at 37 °C for two hours. The reaction was initiated by the addition of FH. It was terminated by the addition of a mixture containing 5 µl 4x NuPAGE LDS sample buffer and 2 µl 10x NuPAGE reducing agent, and by heating the tube at 100 °C for 120 s. The presence of product (*i.e.* cleaved alpha'-chain) was detected by the appearance of bands at 68 and 43 kDa in SDS-PAGE.

2.7 Surface plasmon resonance

All surface-plasmon resonance (SPR) experiments were performed on a BIAcore T200 instrument (GE Healthcare) at 25 °C.

2.7.1 Binding of recombinant murine factor H to human C3b

An experiment was devised to measure binding of rmFH and rmFH-CHA to human C3b (CompTech). The surface of a C1 chip (Series S Sensor Chip C1 with a matrix-free carboxymethylated surface, GE Healthcare) was prepared by immobilizing 258 response units (RU) of C3b (aiming for 250 RU) using standard amine coupling (BIAcore manual). All experiments were performed

using HBSP⁺ buffer (10 mM HEPES, 150 mM NaCl and 0.05% w/v surfactant P20, GE Healthcare).

For each FH protein, concentrations of 0, 0.06, 0.13, 0.25, 0.5, 1 and 2 μ M were each injected twice. An injection/contact time of 180 s was used with a flow rate of 50 μ l/minute and a dissociation time of 300 s. Between sample injections, the chip was regenerated with two injections of 1 M NaCl for 30 s at a flow rate of 50 μ l/minute. Data were analysed using the BIAcore evaluation software and a 1:1 binding model.

2.7.2 Binding of factor H to murine C3b/d

In this experiment, rmFH, rmFH-CHA and mouse FH (from serum) were compared with respect to their binding affinities for murine C3b and murine C3d. Murine C3b was prepared from C3 purified from mouse serum and was a gift from Professor Santiago Rodriguez de Cordoba (Centro de Investigaciones Biologicas, Madrid). The murine C3d was recombinant in origin, and was a gift from Dr Jonathan Hannan (University of Colorado).

The surface of a C1 chip was prepared by immobilizing 242 RU of C3b (aiming for 250 RU) and 68 RU of C3d (aiming for 80 RU) in different flow cells. Further experimental details are provided in Section 2.7.1.

In the same experiment, the binding of hFH to murine C3b was also assessed.

2.7.3 Decay acceleration activity

2.7.3.1 Human C3 convertase and murine factor H

This assay analysed the ability of both recombinant and serum-purified murine FH to accelerate the decay of the human C3 convertase (C3bBb). A CM5 chip (with a carboxymethylated dextran surface, GE Healthcare) was used with a running buffer of HBSP⁺ containing 1 mM MgCl₂. Human C3b, human factor B (FB) and human factor D (FD) were obtained from CompTech.

Using amine coupling, 1137 RUs of C3b were coupled to the flow cell. The C3 convertase was assembled on the chip by flowing a mixture of 500 nM FB and 50 nM FD over the chip surface for 120 s at 10 μ l/minute. An initial dissociation

phase allowed observation of intrinsic convertase decay (Bb loss). A 100 nM solution of rmFH or mFH was then injected (at 10 µl/minute for 120 s), followed by a further dissociation phase. The surface was regenerated between injections with a 30-s injection of 0.1 µM FH (10 µl/minute) and two 30-s injections of 1 M NaCl (10 µl/minute).

2.7.3.2 Murine C3 convertase and factor H

This assay analysed the abilities of murine FH (rmFH, rmFH-CHA and serum-purified FH) and human FH (from serum, CompTech) to accelerate the decay of the murine C3 convertase (C3bBb). Murine C3b, murine FB and human FD were obtained as described above.

Using amine coupling, 1506 RU of murine C3b were immobilised. The C3 convertase was assembled on the chip by flowing a mixture of 500 nM murine FB and 50 nM human FD over the chip surface for 180 s at 10 µl/minute. An initial dissociation phase allowed observation of intrinsic convertase decay (Bb loss). A 40 nM solution of rmFH or mFH was then injected (at 10 µl/minute for 180 s), followed by a further dissociation phase. The surface was regenerated as described in Section 2.7.3.1.

2.7.3.3 Hybrid C3 convertases

In the same experiment as described in Section 2.7.3.2, hC3b was coupled to a separate flow cell to mC3b, with an RU value of 1493. A mixture of human FD, and either human or murine FB, was flowed over the hC3b and mC3b flow cells, allowing formation of hybrid murine/human C3 convertases.

2.7.4 Human Factor H

2.7.4.1 FH binding to PspCN

The PspCN was produced and experiments were performed by Dr Andrew Herbert as published (160). Experiments were performed using HSBP⁺ (2.7.1) supplemented with 50 µM EDTA. Ni²⁺ (~ 50 RU) and PspCN were immobilized on a Biacore NTA chip via N-terminal His⁶-SUMO tags (~ 150 RU). Solutions of plasma-derived FH, rhFH (V62-rhFH) or D1119G-rhFH were flowed over

the chip (180 s, 100 µl/minute) followed by a dissociation phase of 1500 s. As essentially irreversible binding of FH occurred, the chip had to be regenerated between measurements by two 30-s injections of 350 mM EDTA in 1 M NaCl, then 50 mM NaOH, followed by 0.5 % (w/v) SDS. Data were analysed using the BIAcore evaluation software and a 1:1 binding model.

2.7.4.2 Binding of FH and FH:PspCN to C3b and C3d

Human C3b and C3d were amine coupled to two different flow cells of a BIAcore C1 chip, aiming for 250 RU, and 70 RU respectively. Proteins were pre-incubated with a two-fold molar excess of PspCN (or with HSBP⁺ buffer).

2.7.4.2.1 Plasma-FH, V62-rhFH and D1119G-rhFH

This assay was performed by Dr Andrew Herbert (160). Plasma-derived FH (purchased from CompTech); “wild-type” recombinant human (rh)FH with Val at position 62, Tyr at position 402 and Glu at position 936 (V62-rhFH); and mutant rhFH with Gly (instead of Asp) at position 1119 (D1119G-rhFH) were compared. A dilution series was prepared for each of these and samples were injected (in duplicate) for 90 s at 30 µl/minute. Following a 400-s dissociation phase, the chip was regenerated between sample injections by two 30-s injections of 1 M NaCl. Data were analysed using the BIAcore evaluation software and a 1:1 binding model.

2.7.4.2.2 Plasma-FH, V62-rhFH and I62-rhFH

This assay was performed by Dr Eliza Makou (161). Plasma-derived FH (CompTech) was compared to V62-rhFH (as in Section 2.7.4.2.1) and I62-rhFH (*i.e.* with Ile at position 62, Tyr at position 402 and Asp at position 936). In this experiment however, FH solutions were injected for 150 s at 50 µl/minute and a dissociation time of 600 s.

2.7.4.2.3 I62-rhFH, R53H-rhFH and LA-rhFH

Three samples, I62-rhFH, mutant rhFH with His at position 53 instead of Arg (R53H-rhFH), and mutant rhFH with Leu at position 1191 and Ala at position 1197 (LA-rhFH), were compared. Samples were injected in duplicate for 180 s at 50 µl/minute and a dissociation time of 300 s.

2.7.4.3 Decay acceleration activity

2.7.4.3.1 FH and D1119G-rhFH

This assay was performed by Dr Andrew Herbert (160). ~ 500 RU C3b were amine coupled to a BIAcore CM5 chip, then the C3bBb convertase was assembled on the chip by a 120-s 10 µl/minute injection of 500 nM FB and 50 nM FD (CompTech). Formation of C3 convertase (to ~ 150 RU) was monitored, then an initial dissociation phase (200 s) allowed observation of intrinsic decay. Subsequently, 100 nM PspCN (alone), FH (5 or 10 nM) or 5 nM FH:10 nM PspCN was injected (10 µl/min for 120 s), followed by a further dissociation phase. The chip was regenerated between cycles by a 30-s injection of 0.2 µM FH and two 30-s injections of 1 M NaCl.

2.7.4.3.2 Plasma-FH, V62-rhFH and I62-rhFH

This assay was performed by Dr Eliza Makou (161). ~ 500 RU C3b were amine coupled to a CM5 chip then the C3bBb convertase was assembled on the chip by a 180-s 10 µl/minute injection of 500 nM FB and 50 nM FD (CompTech). Formation of C3 convertase (to ~ 150 RU) was monitored, then an initial dissociation phase (240 s) allowed observation of intrinsic decay. Subsequently, 20 nM FH or 20 nM FH:40 nM PspCN was injected (10 µl/minute for 180 s), followed by a further dissociation phase. The chip was regenerated between cycles by a 30-s injection of 0.1 µM FH and three 30-s injections of 1 M NaCl.

2.7.4.3.3 I62-rhFH, R53H-rhFH and LA-rhFH

C3 convertase was assembled as in 2.7.4.3.2, except that protein concentrations used were 40 nM FH with 100 nM PspCN.

2.8 Red cell lysis assays

2.8.4 Haemolysis protection assays

This assay was based on those previously described (162) (with HEPES replacing veronal buffer). Sheep and rabbit whole blood was purchased from TCS Biosciences. Human FH and FH-depleted serum were procured from Complement Technology. The assay was performed in a 96- round-bottomed

well plate. Erythrocytes were prepared by washing three times with buffer E1 (20 mM HEPES, 145 mM NaCl, 0.1% w/v gelatin (porcine skin, Fluka), 10 mM EDTA, pH 7.3) and three times with buffer E2 (20 mM HEPES, 145 mM NaCl, 0.1% w/v gelatin, 10 μ M EDTA, pH 7.3). Erythrocytes were used at a density equating to an absorbance of 1.0-1.2 at 412 nm when fully lysed in water. FH-depleted serum was reconstituted with FH to a final concentration of 1 μ M (with or without 2 μ M PspCN), and then combined with 5 μ l erythrocyte suspension and buffer E2 to a final volume of 45 μ l. Lysis was initiated by addition of 5 μ l 50 mM magnesium ethylene glycol tetra-acetic acid (EGTA), pH 7.3. The plate was incubated at 37 $^{\circ}$ C for 30 minutes then reactions were quenched with 200 μ l buffer E1. The plate was spun at 1500 g for 10 minutes at 4 $^{\circ}$ C (to pellet non-lysed cells), then 100 μ l of supernatant from each well was transferred to a flat-bottomed 96-well plate for reading at A_{412} .

The assay comparing plasma-derived FH with D1119G-rhFH $\pm$ PspCN (Figure 5.6) was performed by Dr Andrew Herbert (160). The assays comparing V62-rhFH and I62-rhFH $\pm$ PspCN (Figure 5.10) and comparing I62-rhFH, R53H-rhFH and LA-rhFH $\pm$ PspCN (Figure 5.16) were performed by Dr Yi Yang (Institute of Cellular Medicine, Newcastle University) (161).

2.8.5 Sheep erythrocyte assays

The following assays were performed by Dr Edwin Wong (Institute of Cellular Medicine, University of Newcastle) using methods previously described (122, 163).

2.8.5.1 Preparation of cells

Sheep erythrocytes (E_S) in Alsever's solution (TCS Biosciences) were pelleted and resuspended three times in complement-fixation diluent (C-FxD) (Oxoid, ThermoFisher Scientific). The washed cells, at 2% (w/v) in C-FxD, were sensitised by incubation with anti- E_S stroma antibody from rabbit (Sigma) (diluted 1:4000 (v/v) in the C-FxD) for 30 minutes at 37 $^{\circ}$ C. Sensitised sheep erythrocytes (EA_S) were washed three times in C-FxD and then resuspended in C-FxD to a final cell density of 2% (w/v). C3b was then deposited on the EA_S surface by adding one volume of 4% (v/v) FB-deficient and FH-deficient normal

human serum ($\Delta B\Delta H$ -NHS) containing 6 $\mu\text{g/ml}$ *Ornithodoros moubata* complement inhibitor (Varleigh Immuno Pharmaceuticals) in C-FxD, followed by a 20-min incubation at 37 °C. $\Delta B\Delta H$ -NHS was made by passing NHS over a 1 ml HiTrap N-hydroxysuccinimide-high-performance sepharose (NHS-HP; Sigma-Aldrich) column with bound monoclonal antibody to FB (JC-1) and a 1 ml HiTrap NHS-HP column with bound monoclonal antibody to FH (OX24).

The C3b-coated EA_S (EA_S -C3b) were washed three times in AP buffer (5 mM sodium barbitone, pH 7.4, 150 mM NaCl, 7 mM MgCl_2 , 10 mM EGTA). Finally, EA_S -C3b cells were resuspended at 2% (v/v) in AP buffer.

2.8.5.2 Cofactor assays on erythrocytes

Cofactor activity was determined by adding (in triplicate) 50 μl from a dilution series of wild-type FH or mutant FH solutions, with or without PspCN at a 2:1 PspCN/FH molar ratio, to 50 μl of a 2% (w/v) suspension of EA_S -C3b in AP buffer containing 2.5 $\mu\text{g/ml}$ FI (CompTech). After 15 minutes (25 °C) of C3b proteolysis, cells were washed three times in 200 μl of AP buffer and pelleted. Washed cells were resuspended in 50 μl of AP buffer in preparation for C3bBb formation as an indirect measure of surviving C3b. C3bBb was formed by adding 50 μl of a solution containing 40 $\mu\text{g/ml}$ FB and 0.2 $\mu\text{g/ml}$ FD (CompTech) to 50 μl of the FH-treated and FI-treated EA_S -C3b and incubating for 15 minutes at 37 °C. Lysis was then used as a read-out of C3bBb formed on cells. This part of the assay was performed by adding $\Delta B\Delta H$ -NHS to a final concentration of 4% (v/v) to the suspended cells and incubating for 20 minutes at 37 °C. To quantify lysis, cells were pelleted, and the absorbance (A_{420}) of the supernatant was measured on a Multiskan Ascent Plate Reader (Agilent). The no-lysis controls, for blank subtraction, contained no FB or FD. Maximal lysis was determined by an A_{420} measurement following the addition of PBS, instead of FH-solution, to the EA_S -C3b. Mean readings from experiments performed in triplicate were calculated. Total protection from lysis for each concentration of FH was determined by subtracting, from one, the following: $((\text{mean } A_{420} \text{ for a particular FH sample}) - (\text{mean } A_{420} \text{ for a no-lysis control sample})) / (A_{420} \text{ for a sample where PBS was used rather than FH})$. This was

then multiplied by 100. A sigmoidal curve was fitted using SigmaPlot and employed to determine the concentration at which FH gave 50% protection from lysis (IC_{50}).

2.8.5.3 Decay-accelerating activities on erythrocytes

C3bBb was formed on EA_s -C3b by adding 50 μ l of AP buffer containing 40 μ g/ml of FB and 0.2 μ g/ml of FD to 50 μ l of a cell suspension (see Section 2.8.2.1). After 15 minutes (37 $^{\circ}$ C), C3bBb formation was stopped by the addition of PBS containing 250 μ M EDTA, pH 7.4, at a 1:20 dilution. Following the addition of 50 μ l from a dilution series of (wild-type or mutant) FH solutions, (with or without PspCN at a 2:1 ratio) in PBS, C3bBb was allowed to decay for 15 minutes (25 $^{\circ}$ C). The extent of lysis then served as a measure of remaining C3bBb, as described above. Mean readings of experiments in triplicate were calculated. Total protection from lysis for each concentration of FH was determined as in Section 2.8.3.2, and a sigmoidal curve was fitted using SigmaPlot to determine the concentration at which FH gave 50% protection from lysis (IC_{50}).

2.9 Recombinant mFH in a murine model of C3 Glomerulopathy

2.9.1 Lipopolysaccharide (LPS) removal

Protein samples were transported to Newcastle University on dry ice, defrosted at 37 $^{\circ}$ C and subsequently kept at room temperature during the LPS-depletion process. The procedure for removal of LPS from FH samples was performed by Dr Jeremy Palmer (Institute of Cellular and Genetic Medicine, Newcastle University) using phase separation with Triton X-114 (164). The endotoxin-depleted protein solution was then applied to Biobeads (Calbiochem) in an attempt to remove residual detergent. Levels of LPS were estimated pre- and post-procedure by measuring pyrogen-induced secretion of interleukin 6 (IL-6) on Mono Mac 6 cells. This assay has been shown to be equivalent to the more widely recognised assay for LPS detection, the Limulus Amoebocyte Assay (165). A second LPS depletion procedure was subsequently applied to ensure complete LPS removal (based on the

experience of Dr Palmer). Proteins were spin-filtered (Millipore 0.2 μ M sterile filter) before confirmation of protein concentration (using a NanoDrop ND1000, ThermoFisher and an extinction coefficient of 230870), prior to transportation to Imperial College, London. For Experiment One, proteins were transported at room temperature. For Experiment Two, proteins were transported on dry ice, stored at -80 $^{\circ}$ C, and defrosted prior to use.

2.9.2 *CFH*^{-/-} mice

CFH^{-/-} mice were generated as previously described (119) and backcrossed onto the C57BL/6 genetic background. Mice used were 7-9 weeks old. All experimental procedures were conducted in accordance with institutional guidelines (Imperial College, London). Blood samples were collected into EDTA solution *via* tail venesection, then plasma was obtained using centrifugation prior to storage at -80 $^{\circ}$ C. Mice were culled at indicated time points, and the kidneys were collected into PBS and snap frozen in OCT embedding matrix (CellPath) for storage at -80 $^{\circ}$ C.

Handling of *CFH*^{-/-} mice and injection of proteins/blood sampling/collection and processing of kidneys for Experiment One and Two was kindly performed by Dr Talat Malik (Centre for Complement and Inflammation Research, Imperial College London). Dr Malik performed statistical tests using GraphPad Prism 8.0. Subsequent assays were performed by Heather Kerr unless otherwise stated.

2.9.3 Experiment One

Four *CFH*^{-/-} mice were injected (intra-peritoneal, *i.p.*) with 0.5 mg rmFH, four mice were injected with 1 mg rmFH, two mice were injected with native mFH (*i.e.* mFH from serum) and two mice were injected with PBS (Endotoxin-Free, Sigma). Mice were bled pre-injection, at six hours and at 24 hours post-injection. Mice were culled at 24 hours.

2.9.4 Experiment Two

Five *CFH*^{-/-} mice were injected *daily* (*i.p.*) with 0.5 mg rmFH, two mice were injected *daily* with 0.4 mg rhFH (*i.e.* I62-rhFH; 0.4 mg was used due to the

limited amount of concentrated protein available at the time of experiment), and five mice were injected *daily* with PBS, for ten days in total. Mice were bled pre-injection, at 24 hours, five days and 11 days (*i.e.* 24 hours after final injection). Mice were culled at 11 days post-initial injection.

2.9.5 ELISA

2.9.5.1 FH

Murine FH levels were measured by capture with a sheep anti-hFH cross-reacting antibody (ABIN 113017) followed by detection with a cross-reacting goat anti-hFH biotinylated antibody (Quidel A312, biotinylated by Dr Talat Malik) and a streptavidin alkaline phosphatase (BD Pharmingen) reporter. Results were quantified by reference to a standard curve of rmFH protein. Human FH levels were measured by capture with an anti-FH monoclonal antibody (OX24, Thermo MA170057) and detection with a polyclonal sheep anti-human FH antibody (peroxidase-conjugated) (Jackson Lab: 713-035-147) and TMB (tetramethylbenzidine). Results were quantified by reference to a standard curve of rhFH protein. ELISAs were performed by Dr Hannah Lomax-Brown (Centre for Complement and Inflammation Research, Imperial College London).

2.9.5.2 C3

C3 levels were measured by ELISA using goat anti-mouse C3 antibody (MP Biomedicals) as previously described (110). Results were quantified by reference to a standard curve generated from acute-phase sera containing a known quantity of C3 (Calbiochem). For Experiment One, C3 ELISA were performed by Dr Talat Malik.

2.9.5.3 FH antibody

Murine anti-FH antibodies were detected by an ELISA. Nunc MaxiSorp ELISA plates were coated with 0.0625 µg of either rmFH or rhFH that had been diluted in 50 mM sodium carbonate, pH 9.6. After washing and blocking with 1% BSA in PBS, the plasma samples to be tested were added at a dilution of 1:50 and incubated at room temperature for one hour. After washing, the plates were incubated for a further hour at room temperature with a goat anti-murine IgG

antibody labelled with alkaline phosphatase (Sigma A3438). After additional washing, enzymatic activity was revealed using SIGMA-FAST p-Nitrophenyl phosphate tablets. Antibody titres were expressed as arbitrary ELISA units calculated as optical density of the sample x 50.

2.9.6 Glomerular immunostaining

Cryosections (μm) from snap-frozen kidneys were fixed in acetone for ten minutes. C3 was detected with FITC-conjugated goat anti-mouse C3 antibody (MP Biomedicals). For C3d detection a biotin-blocking system (Dako) was applied prior to detection using a biotinylated goat anti-mouse C3d antibody (R+D systems) followed by Avidin-Alexa 488 (Invitrogen). IgG was visualized with a FITC-conjugated goat anti-mouse IgG antibody (Sigma). Twenty glomeruli were visualized per section and fluorescence intensity expressed in arbitrary units.

2.9.7 Western Blot for C3

Plasma samples were separated using electrophoresis on a 10% polyacrylamide gel under denaturing and reducing conditions. For detection, goat anti-serum to mouse C3 (MP Biomedicals) was used with visualisation *via* HRP conjugated anti-goat IgG (Sigma). Blots were visualized using Pierce ECL Western Blotting substrate (Thermo Scientific).

2.9.8 Experiment 3

This experiment was performed by Dr Yi Yang and Dr Kevin Marchbank (Institute of Cellular Medicine, University of Newcastle). Animals (8 weeks old wild-type and *CFH*^{-/-}; or >4.5 months old *CFH*^{-/-}) received 20 μg of either mFH or rmFH protein in sterile PBS *via i.p.* injection on Day 0 and Day 28. Animals were bled *via* tail vein venesection into 10 mM EDTA/PBS every seven days until day 41 when they were terminally exsanguinated under anaesthesia. To detect murine anti-FH antibodies, Nunc maxiSorp ELISA plates were coated with 5 $\mu\text{g}/\text{ml}$ (50 μl per well) of either rmFH or native mFH diluted in PBS (Lonza, UK). After washing and blocking with 0.1% Tween in PBS, the plasma to be tested was added at a dilution of 1:100 in triplicate wells and incubated

for one hour (room temperature). After washing, the plates were incubated for a further hour (room temperature) with sheep anti-murine IgG antibody labelled with horse radish peroxidase (1/2000 dilution, Jackson ImmunoResearch). After additional washing, enzymatic activity was revealed by addition of ready-made TMB solution (Leinco, via Universal Biologicals LTD, UK) and the assay was stopped after using an equal volume of 10% v/v H₂SO₄. Absorbance at 450 nm was measured using a Titretek plate reader. The means of triplicate readings are shown. For a positive control, a mouse anti-FH monoclonal antibody (mAb 2a5, gift from Professor Claire Harris), was diluted 1/250,000 or 1/750,000. For a negative control, PBS alone was used.

To compare the immune-reactivity of mouse terminal bleed sera generated with antigens of different origin, an ELISA protocol was performed as above, with either native mFH or rmFH coated to the plate.

3: Production and purification of Factor H

3.1 Recombinant Murine Factor H

3.1.1 Recombinant murine factor H production in KM71H cells transformed with pPICZαB containing GeneArt DNA

As discussed previously, recombinant human FH (rhFH) had been produced in *P. pastoris* strain KM71H using the expression vector pPICZαB (23). A subsequent attempt to produce recombinant murine FH (rmFH) from an expression-optimised gene (GeneArt) inserted into pPIC3.5K and using the *P. pastoris* strain GS200 did not succeed (personal communication – Fern Slingsby). This construct utilised a mouse secretion-signal peptide, rather than the yeast alpha-factor secretion-signal peptide. In the current work it was decided to re-clone the expression-optimised rmFH gene into pPICZαB (that incorporates DNA coding for the yeast alpha-factor secretion-signal peptide) prior to transformation into strain KM71H.

3.1.1.1 DNA manipulation and PCR screening

A PCR was used to amplify the expression-optimised rmFH-coding DNA sequence from pPIC3.5K and to insert cleavage sites for *Pst*I and *Xba*I (Section 2.1.1.1). A restriction-enzyme digest was then performed using *Pst*I and *Xba*I, removing the additional six base-pairs derived from the PCR (Section 2.1.1.3). Ethanol-precipitated DNA was subsequently inserted into pPICZαB prior to transformation of *E. coli* cells and overnight growth on an agar plate. Eight colonies were screened for successful insertion of rmFH DNA into pPICZαB using PCR (Section 2.1.1.8) (Figure 3.1). Two colonies showed a DNA band of the expected size of ~4 kilobase-pairs (kbp). Subsequent full sequencing showed that colony number 5 had the correct sequence and this was used for a maxi-prep. The maxi-prepped vector containing the insert was linearized with *Sac*I prior to transformation into *P. pastoris* KM71H cells.

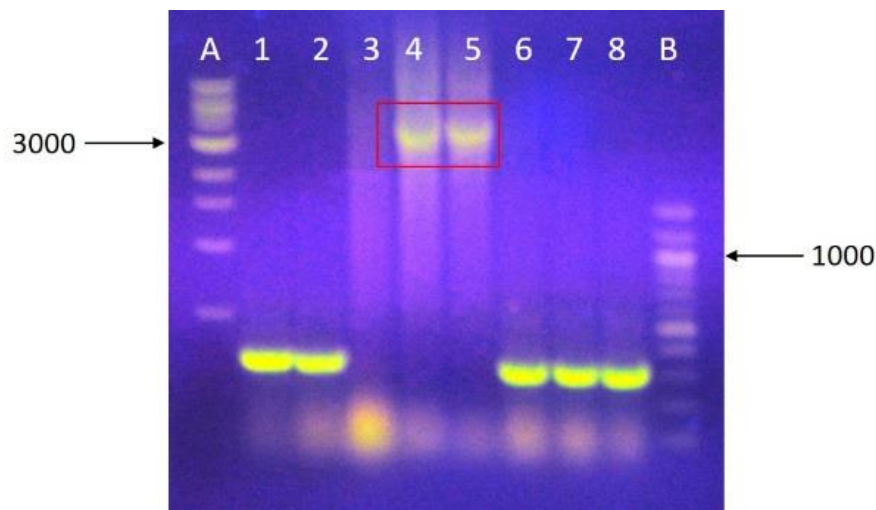


Figure 3.1 Outcome of PCR to test eight colonies for insertion into pPICZαB of DNA coding for rmFH. Analysis of DNA from colonies 4 and 5 shows a band (red box) of the expected size ~ 4 kbp. A: 1-kbp ladder, B: 100-bp ladder

3.1.1.2 Test expressions

pPICZαB utilises the Zeocin antibiotic-resistance gene for selection of successfully transformed colonies. In theory, the higher the Zeocin concentration tolerated, the greater the number of Zeocin-resistance gene insertions into the *P. pastoris* genome and hence also the higher the copy number of the rmFH gene. An initial attempt at transformation yielded a few colonies on both 200 µg/ml and 300 µg/ml (final concentration) Zeocin plates.

Small-scale protein-production trials were conducted in 25 ml cultures grown from three single colonies picked from each of the two plates. The outcome was assessed by enzymatically deglycosylating proteins secreted into the medium and then running them on SDS-PAGE. Based on previous experience of rhFH, bands from deglycosylated rmFH were expected to migrate at 100 kDa (*i.e.* at the same position as a 100-kDa marker protein) under non-reducing conditions and at 150 kDa under reducing conditions. However, Coomassie Blue staining revealed no such bands (Figure 3.2a).

Despite the lack of Coomassie staining, Western blot using an anti-FH antibody (Santa Cruz) (Figure 3.2b) showed bands of approximately the expected mass, in lanes run under reducing condition, arising from two of the cultures.

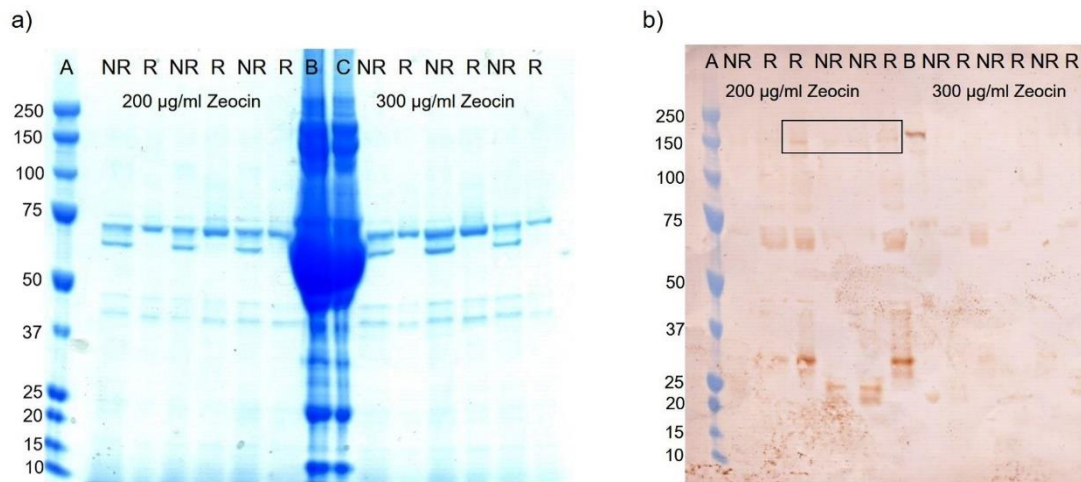


Figure 3.2 Protein-production trials following initial attempt at transformation of KM71H cells with pPICZαB containing a gene for rmFH. a) SDS-PAGE. Three colonies from 200 µg/ml and three from 300 µg/ml Zeocin agar plates were cultured, and samples of growth media after spinning out cells were run under non-reducing (NR) or reducing (R) conditions. All wells were loaded with 10 µl supernatant. A: protein molecular weight markers (kDa); B: mouse serum diluted 1:5 and run under R conditions; C: mouse serum diluted 1:10 and run under R conditions. The band at ~70 kDa is Endo H_r. b) Western blot. Lanes as per a). Primary antibody: rabbit anti-mouse FH diluted 1:200 (Santa Cruz); Secondary antibody: goat anti-rabbit IgG HRP diluted 1:1000 (Cell Signalling Technology).

A further *P. pastoris* transformation was attempted to provide more colonies and additional protein-production trials were conducted. Figure 3.3a shows the results of SDS-PAGE after concentration of supernatant (30-kDa MWt cut-off Vivaspinn concentrator) from an initial volume of ~25 ml down to ~1 ml. The presence of multiple bands after four days of incubation suggest that some cell lysis had occurred, and it is difficult to judge if any of these correspond to secreted rmFH. A Western blot (Figure 3.3b) provided no clear evidence for significant amounts of secreted intact rmFH. The multiple bands observed in the Western blots of Figures 3.2b and 3.3b could either be degradation products of rmFH or false positives. To test this some growths of

untransformed KM71H stocks (*i.e.* no recombinant plasmid present) were analysed in the same way. Figure 3.4 shows that the concentrated supernatants yielded patterns of bands that are very similar to those observed for the transformed strains. This observation suggested that either the primary or secondary antibody used in the Western blot are cross-reacting with native *P. pastoris* proteins released into the growth medium either through secretion or low-level cell lysis.

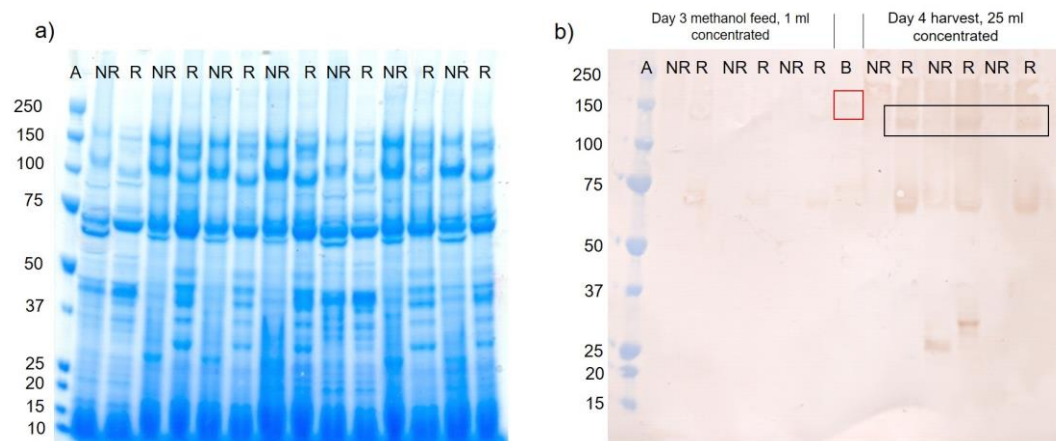


Figure 3.3 Further small-scale protein-production trials. a) SDS PAGE. Seven colonies from 300 µg/ml Zeocin plates were cultured for four days post-induction and then aliquots of supernatant taken for analysis. The supernatant was concentrated from 25 ml to 1 ml. b) Western blot for three cultured colonies at three or four days post-induction, concentrated prior to loading as indicated. Samples were taken each day of the methanol feed. A: protein MWt markers (kDa); B: mouse serum diluted 1:10 and run under R conditions. The antibodies used were as in Figure 3.2b. All wells were loaded with 10 µl sample. NR = non-reduced; R = reduced.

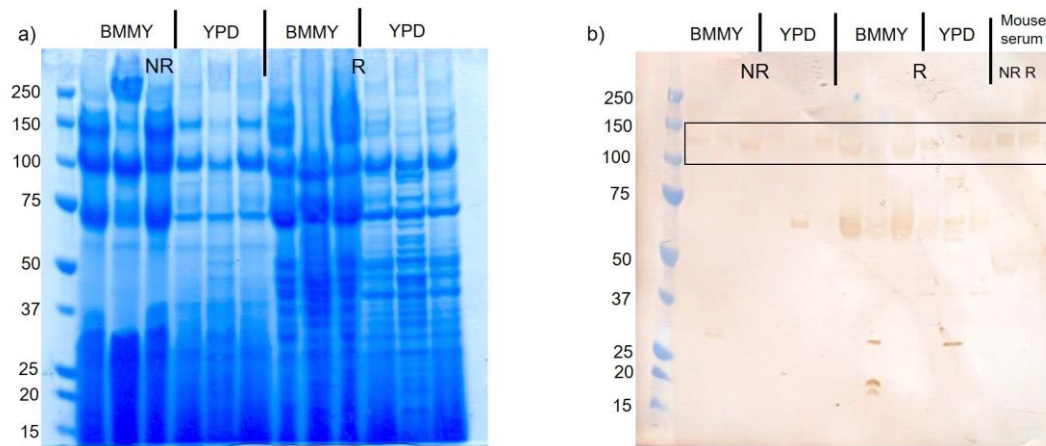


Figure 3.4 Small-scale protein-production trials (negative control) for untransformed KM71H cells. a) SDS-PAGE. Colonies grown in BMMY or YPD media. Supernatant concentrated from 25 ml to 1 ml. b) Western blot. Colonies as in a). Mouse serum was diluted 1:10. A: MWt markers. Antibodies were as in Figure 3.2b. BMMY = buffered methanol-complex medium YPD = yeast peptone dextrose medium. All wells were loaded with 10 μ l sample. Box indicates yeast protein bands of a MWt similar to mFH from mouse serum.

To ascertain whether the primary or secondary antibody was cross-reacting, a Western blot was performed using only the goat anti-rabbit IgG HRP (Figure 3.5). The lack of bands suggests that yeast proteins likely cross-react with the rabbit anti-mouse FH used as a primary antibody.

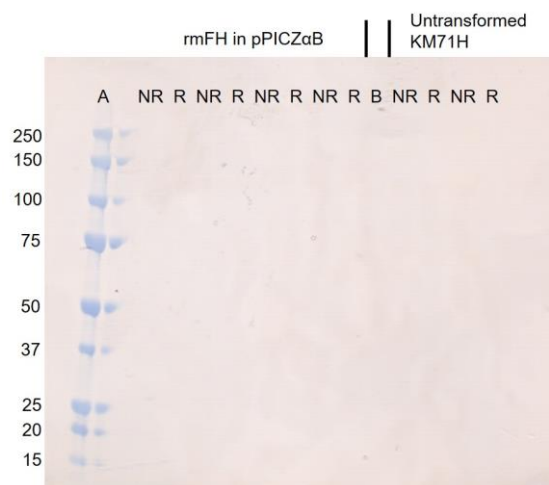


Figure 3.5 Western blot using secondary antibody alone. Supernatant from rmFH in pPICZ α B colonies or untransformed KM71H. A: protein MWt markers. All wells were loaded with 10 μ l supernatant. B: mouse serum diluted 1:10 run under R conditions. Secondary antibody: goat anti-rabbit IgG HRP diluted 1:1000 (Cell Signalling technology).

An alternative specific detection method for rmFH was required. Due to high sequence similarity between human and mouse FH, it was judged that anti-human FH polyclonal antibodies may cross-react with mouse FH. To test this, a Western blot was performed using goat anti-human FH antibody (Calbiochem) to analyse mouse serum, supernatant from small-scale rmFH-production trials and untransformed KM71H cells (Appendix D). Figure 3.6 shows that the anti-hFH antibody detects non-reduced mFH in mouse serum, with only minimal cross-reaction against *P. pastoris* proteins. Unfortunately, there was still no evidence of rmFH production.

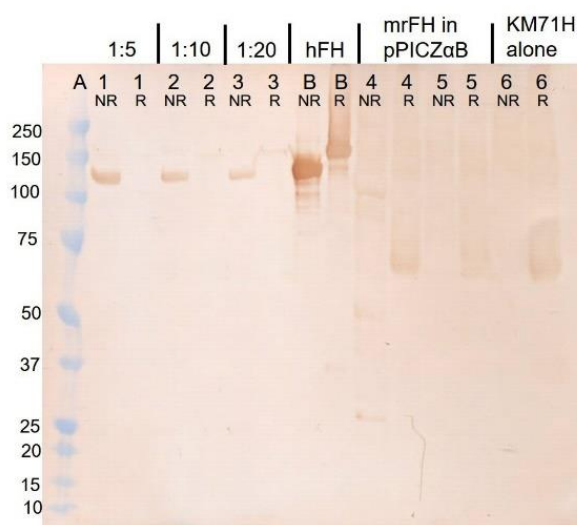


Figure 3.6 Western blot using anti-human FH primary antibody. Lanes labelled 1-3, mouse serum at 1:5, 1:10 and 1:20 dilutions, alternately run under NR/R conditions; Lanes labelled 4-5, supernatants from rmFH-production trials, alternately run under NR/R condition; Lane 6, supernatants from cultures of untransformed KM71H cells, run under NR/R conditions. All wells were loaded with 10 µl sample. A: MWt markers; B: 2 µl 1mg/ml hFH isolated from serum (CompTech). Primary antibody, goat anti-human FH diluted 1:200 (Calbiochem); Secondary antibody, rabbit anti-goat IgG HRP diluted 1:50,000 (Sigma).

3.1.2 Recombinant murine FH production in KM71H cells transformed with pJe912 containing DNA2.0 DNA

3.1.2.1 Small-scale test production

An alternative expression-optimised gene was designed and purchased (DNA2.0) that shared 80% identity with GeneArt's DNA. The new gene was supplied in a maxi-prepped *P. pastoris* vector pJe912 (vector map Appendix B). Like pPICZαB, this vector utilises Zeocin resistance for positive selection and contains the DNA for the alpha-factor secretion-signal.

The new rmFH-containing plasmid was linearised by *Bam*HI or *Swa*I and transformed into *P. pastoris* KM71H cells. More positive colonies were obtained from the *Bam*HI-linearised plasmids, and these were selected and used for subsequent small-scale production tests. Figure 3.7 shows the results of analysis by SDS-PAGE (Fig. 3.7a) and Western blot (Fig. 3.7b), with samples run alongside a “positive” control, namely supernatant from a previously prepared strain of *P. pastoris* that produces recombinant human FH (rhFH).

These samples were not concentrated prior to SDS-PAGE, and no obvious rmFH bands can be seen migrating at the predicted MWts of 100 kDa (non-reduced) or 150 kDa (reduced) in the Coomassie blue-stained gel. Bands of the expected MWt for rhFH were detected (non-reduced) on the Western blot (boxed in Figure 3.7b).

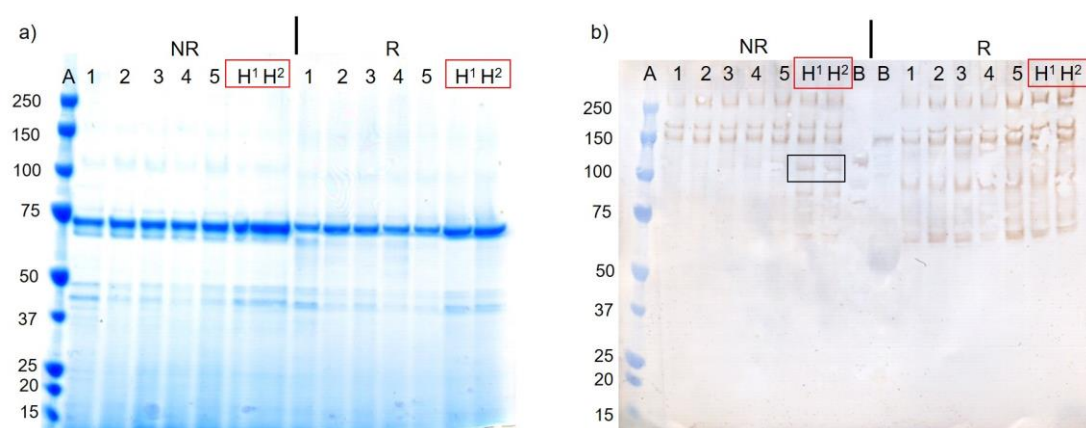


Figure 3.7 Small-scale production trials for rmFH using the DNA2.0 gene. a) SDS-PAGE. Lanes 1-5, colonies as numbered, run under R or NR conditions. b) Western blot. Lanes as in Figure 3.7 a). Lanes H¹H², rhFH. All wells were loaded with 10 µl sample. A: protein MWt markers; B: mouse serum diluted 1:5. Antibodies as in Figure 3.6b). The band at ~70 kDa is Endo H_f.

3.1.2.1.1 Small-scale purification

Precedent, and Figure 3.7a, suggested that lack of detectable FH in crude supernatant from small-scale shaker-flask growths does not preclude the possibility of this strain subsequently yielding useful amounts of protein in a fermenter. To explore this possibility, an attempt was made to enrich rmFH

from a small-scale production trial using a HiTrap 1.0-ml heparin-affinity column (Section 2.2.5.1.1), thereby taking advantage of the known affinity of hFH for polyanions (Figure 3.8). High-salt eluted fraction numbers 3-5 showed Western blot-positive bands that are consistent with non-reduced rmFH at ~ 100 kDa, and reduced rmFH at ~ 150 kDa. This was the first convincing evidence for production of rmFH.

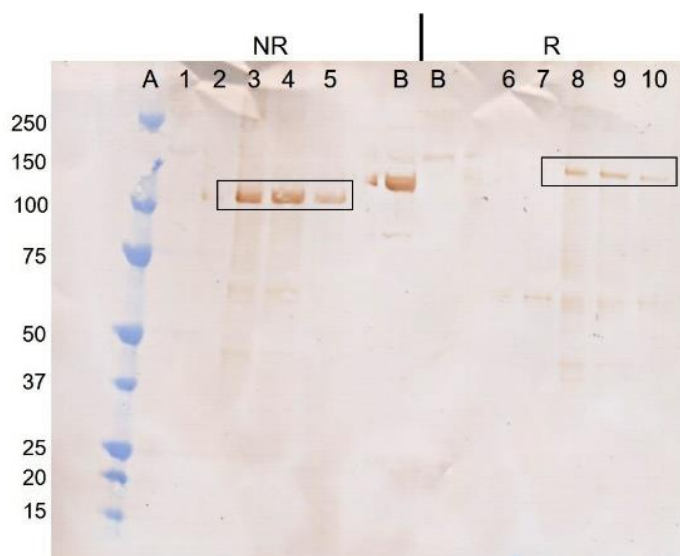


Figure 3.8 Western blot indicates the success of a rmFH capture on a heparin column. Lanes 1-5, high-salt eluted fractions run under NR conditions; lanes 6-10, high-salt eluted fractions under R conditions. Each well was loaded with 10 μ l of the fraction. A: protein MWt markers; B: mouse serum diluted 1:5 run under NR and R conditions. Antibodies were as in Figure 3.6b). Boxes outline protein bands at the expected MWt for rmFH.

3.1.2.2 Fermentations

Building on this encouraging evidence of rmFH production from KM71H cells transformed with pJe912, a series of large-scale growths were performed in fermenters. Table 3.1 summarises the starter cultures and fermentation conditions. The colony chosen for each fermentation had initially grown on a YPD plate containing 500 μ g/ml Zeocin. It was re-streaked on a Zeocin-containing YPD plate and incubated for 3-5 days, prior to picking a single colony for inoculation of a 10-ml starter culture that contained various concentrations of zeocin.

Fermentation	Zeocin concentration of starter colony ($\mu\text{g/ml}$)	Zeocin concentration of inoculation culture ($\mu\text{g/ml}$)	Fermentation size (litres)	Additional conditions
1	500	1000	5	Standard 5L
2	500	500	5	Low basal salts, PMSF, casamino acids
3	500	500	10	Standard 10L
4	500	500	10	Low pH
5	500	1000	10	Standard 10L

Table 3.1 Summary of rmFH pJe912 in KM71H fermentation conditions

3.1.2.2.1 Fermentation One

For this fermentation, the starter colony was re-streaked on a YPD plate containing 2 mg/ml Zeocin, motivated by the theory that the higher the concentration of Zeocin a colony tolerates, the higher the number of insertions, *i.e.* copy numbers of the Zeocin-resistance gene and the rmFH-encoding DNA (166-170).

A pump was used to deliver 50 ml methanol, initially, and every six hours after induction. The gap between feeds was then gradually reduced over the methanol-feeding period depending on the status of the yeast as judged by their demand for oxygen, as oxygen is required for the first step in methanol catabolism. An increase in stirring of the media maintained the dissolved oxygen concentration. A slow increase in oxygen consumption, and hence agitation rate, implied the yeast cells are growing slowly and may be in a sub-optimal state.

The goal was to ensure the cells had used up each batch of methanol prior to the next feed, and hence to reduce the risk of methanol toxicity. This method had previously been found to be successful in the production of rhFH (personal communication, Andrew Herbert) in the laboratory.

Experience, within the group, of previous fermentations of *P. pastoris* strains producing shorter recombinant human FH fragments had led us to expect a brown, relatively clear supernatant after spinning out the cells. However, in this case, the crude supernatant remained green in colour and cloudy despite prolonged centrifugation. This suggested lysis or bacterial contamination. Samples were taken of crude supernatant for SDS-PAGE and Western blot, then a 50-ml sample was diluted 1:5 and run through a heparin-affinity column (as in 3.1.2.1.1).

Figure 3.9 shows the results of Coomassie Blue staining and Western blot of SDS-PAGE gels. Protein bands corresponding to rmFH could not be detected in the crude supernatant, but there are candidate Coomassie Blue-stained bands visible in lanes corresponding to the high-salt eluted (and therefore concentrated) fractions from the heparin-affinity column and these were positive by Western blot. Unfortunately numerous other bands were also detected by Western blot, suggesting low specificity. Both Coomassie Blue staining and Western blot featured a band at ~100 kDa under reducing conditions only (arrow). It was postulated that this band corresponds to a 'clipped' species of rmFH that has been proteolytically cleaved between two cysteine residues that share a disulphide bond.

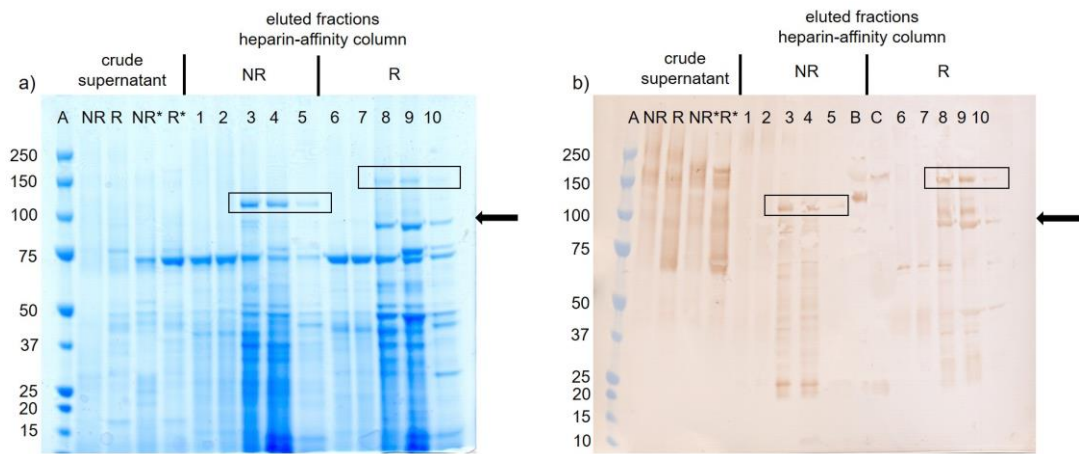


Figure 3.9 Results of Fermentation One a) SDS-PAGE analysis of crude supernatant (before and after* deglycosylation with EndoHf (this enzyme is visible as a band at ~70 kDa) and (lanes 1-10) high-salt eluted fractions from a heparin-affinity column (after deglycosylation). b) Western blot after SDS-PAGE. Antibodies were as in Figure 3.6b). Lane A, MWt markers; Lanes B and C, mouse serum diluted 1:5 run under reducing (R) and non-reducing (NR) conditions. B) Lanes 1-10 as in 3.9a). All wells were loaded with 10 μ l of sample.

The crude supernatant was subjected to cation-exchange chromatography on a SP-Sepharose column and the outcome analysed by SDS-PAGE (Figure 3.10).

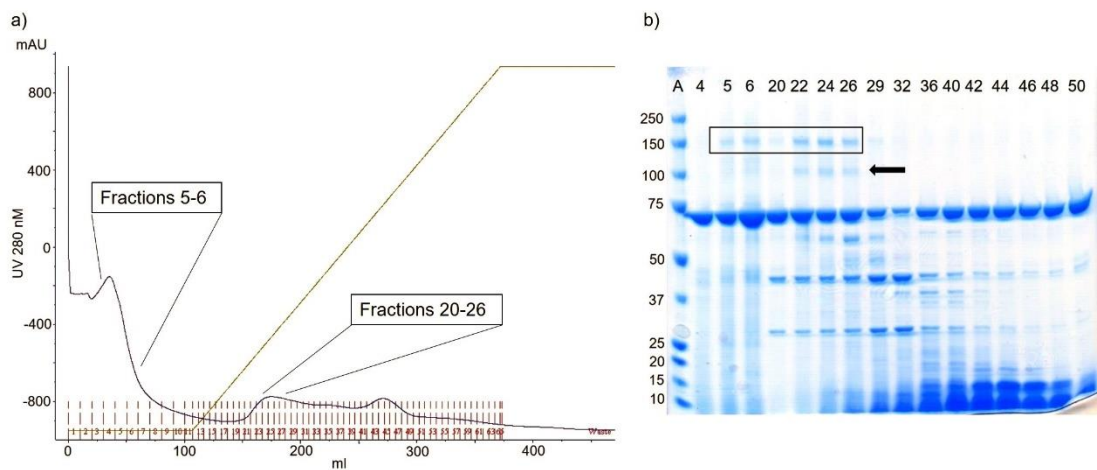


Figure 3.10 Fermentation One: results of SP-Sepharose chromatography. a) Chromatogram from a 0.1 M-1 M NaCl gradient applied over five column volumes. b) SDS-PAGE. The box shows the expected positions of bands corresponding to rmFH (reduced) and the arrow shows the suspected 'clipped' protein. The prominent bands at ~70 kDa are Endo Hf. All wells were loaded with 10 μ l supernatant. A: protein MWt markers.

Relatively weak SDS-PAGE bands corresponding to intact rmFH were obtained for those fractions (20-26) corresponding to the main peak in the chromatogram, despite the concentrating effects expected from cation-exchange chromatography. RmFH seemed to be present in fractions 5 and 6 along with some clipped protein.

This yield of rmFH was only a small fraction of that obtained previously for rhFH (23). Yet the original colony had looked promising in small-scale trials. To test the merits of our decision to increase concentrations of Zeocin (from 0.5 mg/ml to 2 mg/ml) when re-streaking this colony for fermentation and producing the inoculation culture, a small-scale shake-flask growth of the colony used for fermentation was performed. After purification using the procedure described in 3.1.2.1.1, a comparison was possible between the colony grown on low-Zeocin agar and the re-streaked colony grown on high-Zeocin agar. Unexpectedly, the colony which was grown in a higher concentration of Zeocin produced less rmFH (Figure 3.11).

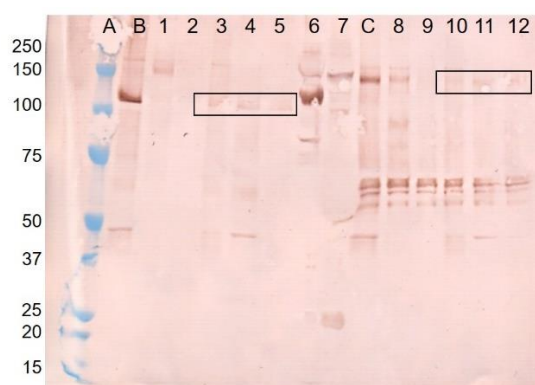


Figure 3.11 Western blot to reveal outcome of another small-scale purification. Lanes 1-5, eluted fractions under non-reducing conditions; lanes 8-12, eluted fractions under reducing conditions. Lanes B/C, fraction 3 from Figure 3.8, under both non-reducing and reducing conditions. All wells were loaded with 10 µl sample. Lanes 6/7, mouse serum diluted 1:5. Antibodies were used as in Figure 3.6b). A: protein MWt markers.

3.1.2.2.2 *Fermentation Two*

This five-litre fermentation employed a growth medium containing low basal salts, addition of casamino acids and use of phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. The use of a low-basal salts medium has been

shown to increase cell viability and heterologous protein production in circumstances where the osmotic pressure of a standard-basal salt medium was apparently detrimental to growth (171). Addition of casamino acids (172, 173) is thought to act as an alternative and competing substrate for degradative proteases. PMSF inhibits serine proteases (174, 175) and acts to reduce degradation of secreted proteins by intracellular proteases. Note that limited availability of the fermenter prevented an optimisation strategy in which only one condition would have been changed at a time. A single colony was picked from the same 500 µg/ml YPD plate as used in Fermentation One but it was used to inoculate a 500 µg/ml starter culture.

The methanol feeding routine was set-up as in Fermentation One, but intervals between feeds were extended to 8-10 hours because the oxygen demand (that reflects methanol metabolism) was slower to increase and took longer to return to baseline after each feed.

The pH is normally maintained at 5.0 in *P. pastoris* fermentation runs (as in Fermentation One) by the addition of ammonium hydroxide thus counteracting the acidifying effect of growth and metabolism in the culture. But in this fermentation, the pH tended to rise towards 6.5, postulated to be due to the addition of casamino acids. This occurred overnight when the fermenter was unattended. It was subsequently controlled by manual addition of 5 M hydrochloric acid. The persistence of a higher-than-usual pH for several hours may have contributed to the slower than expected growth of the yeast.

Aliquots of the fermenter culture were taken daily during methanol feeding and at the time of harvest. Following treatment with EndoH_f, SDS-PAGE allowed a fruitless search for evidence of rmFH production in crude supernatant (data not shown). The three litres of supernatant were treated as per protocol (2.2.8) and applied to a cation-exchange (SP-Sepharose Fast Flow XK 26/20) chromatography column. A NaCl gradient was run from 50 mM to 1 M (instead of starting from 100 mM NaCl as used previously) in an attempt to reduce the amount of rmFH in the flow-through. Figure 3.12 shows the corresponding chromatogram and the results of SDS-PAGE.

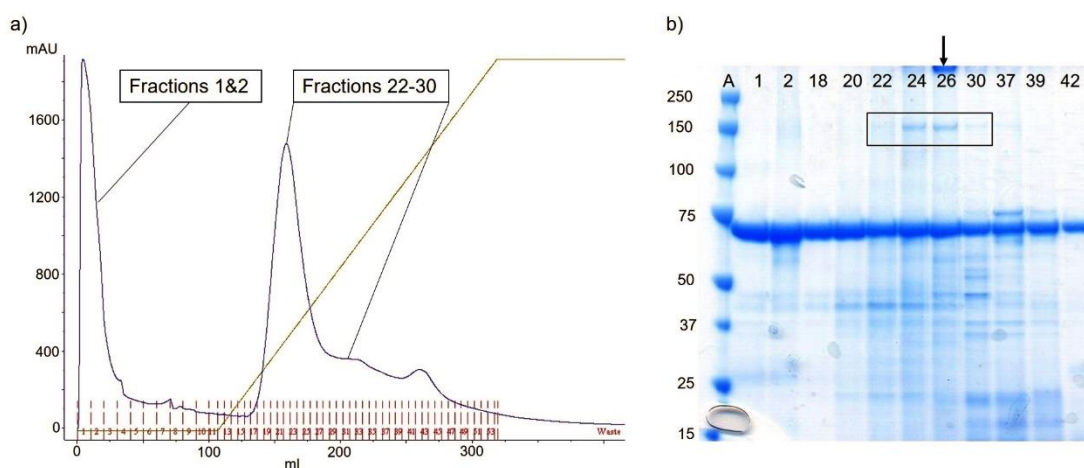


Figure 3.12 Fermentation Two: results of chromatography on SP-Sepharose column a) Chromatogram from a 0.05 M - 1 M NaCl gradient applied over four column volumes. b) SDS PAGE: box shows band expected for rmFH (reduced); arrow shows deposition of material in well. Band at ~70 kDa is Endo H_f. All wells were loaded with 10 µl samples. A: protein MWt markers.

Despite an initially encouraging chromatogram (Figure 3.12), with a large peak corresponding to where rmFH would be expected to elute based on Fermentation One (~200 mM NaCl), the Coomassie-stained SDS-PAGE gel shows weak protein bands for rmFH in fractions centred on fractions 24-26. This material was not further purified.

Fermentation Two was repeated, with almost identical results. Therefore the use of low basal salts and casamino acids was abandoned.

3.1.2.2.3 Fermentation Three

Fermentation Three was a ten-litre fermentation in a standard basal-salts medium. A 500 µg/ml Zeocin starter culture was used. An addition of 100 g (1% w/v) sorbitol and 30 g (0.3% w/v) tryptone was made with each 150-ml methanol feed. The use of sorbitol as a co-substrate with methanol has been found to reduce oxygen consumption and to increase protein production (176, 177). Tryptone is an additional nitrogen source that has been shown to increase cell growth and productivity (178). In total, 2.35 litres of methanol, 1.5 kg of sorbitol and 580 g of tryptone were added to the fermentor. The final volume at the time of harvesting was 14 litres, with 6 kg of cells and 7.5 litres

of supernatant. The supernatant post-centrifugation was less cloudy than in the previous two fermentations, although it remained green in colour.

Figure 3.13 shows the chromatogram and results of SDS-PAGE from the initial SP-Sepharose chromatographic step.

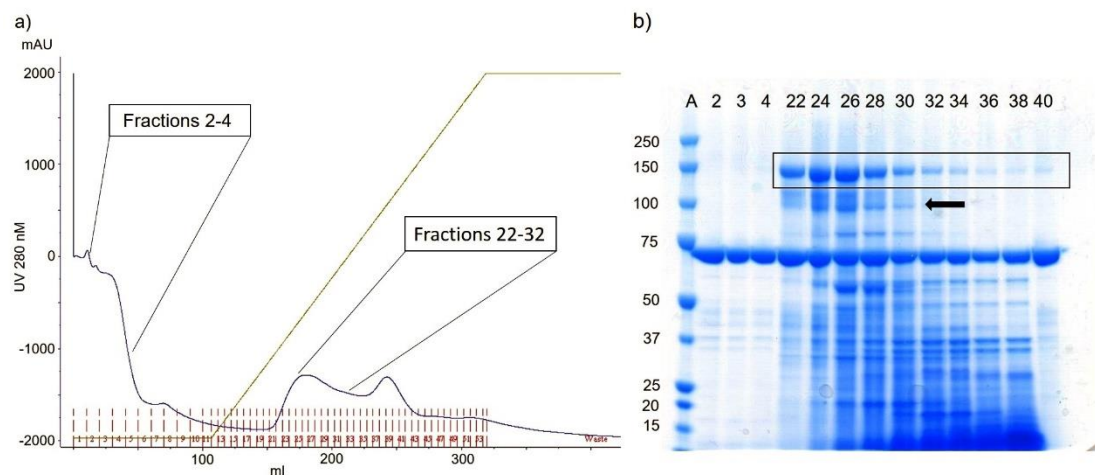


Figure 3.13 Fermentation Three: results of chromatography on SP-Sepharose column. a) Chromatogram from a 0.05 M – 1.0 M NaCl gradient applied over four column volumes. b) SDS-PAGE. The box shows expected positions of bands corresponding to rmFH (reduced). The arrow indicates bands likely to correspond to proteolytically clipped rmFH. Band at ~70 kDa is Endo H_f. Each lane was loaded with a 10 µl aliquot from the indicated fraction. A: protein MWt markers.

There was no detectable rmFH in the flow-through (fractions 2-4), presumably as a result of starting the elution gradient from 50 mM NaCl instead of 100 mM. Relatively (compared to previous fermentations) high quantities of rmFH are present in those fractions corresponding to the main peak and shoulder of material eluting during the salt gradient. Since the gel was run under reducing conditions, the presence of lower-MW “shadow” bands suggests co-purification with clipped proteins.

Fractions 22-32 were pooled (55 ml in total) and subjected to exhaustive Endo H_f treatment. The protein solution was then applied to a lectin-affinity column to remove any protein that still retained glycans.

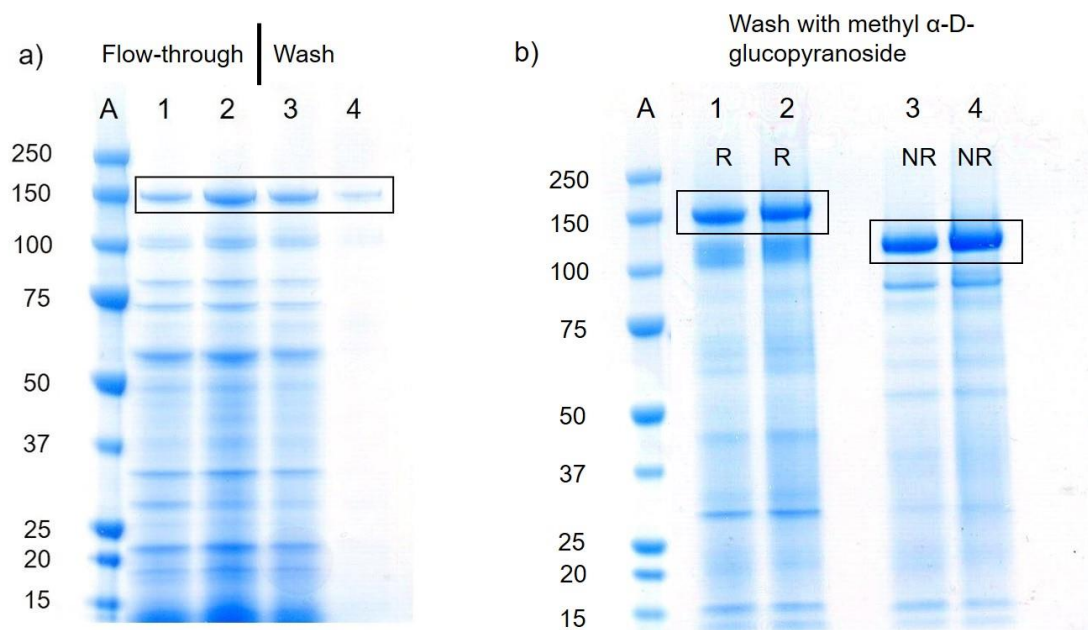


Figure 3.14 Fermentation Three: results of lectin-affinity chromatography. SDS-PAGE a) Lanes 1-2, deglycosylated protein from flow-through; lanes 3-4, two additional wash fractions. All were run under reducing conditions. b) Fractions after elution with 1 M methyl α -D-glucopyranoside. Lanes 1-2, reduced. Lanes 3-4, non-reduced. All lanes were loaded with 20 μ l samples. A: protein Mwt markers. Box: presumed rmFH.

While some protein was present in the flow-through and wash as expected, Figure 3.14 b) shows that most of it eluted only after washing the column with 1 M methyl α -D-glucopyranoside (in 20 mM potassium phosphate, pH 6.0, 200 mM NaCl). This was presumed to be rmFH that had not been fully deglycosylated. Despite these losses, fractions 1-4 (Figure 3.14 a) were pooled (70 ml) and buffer-exchanged into the equilibration buffer for a subsequent chromatographic step on an anion-exchange column (Source Q Tricorn 10/100). Figure 3.15 shows the resultant chromatogram and SDS-PAGE gel.

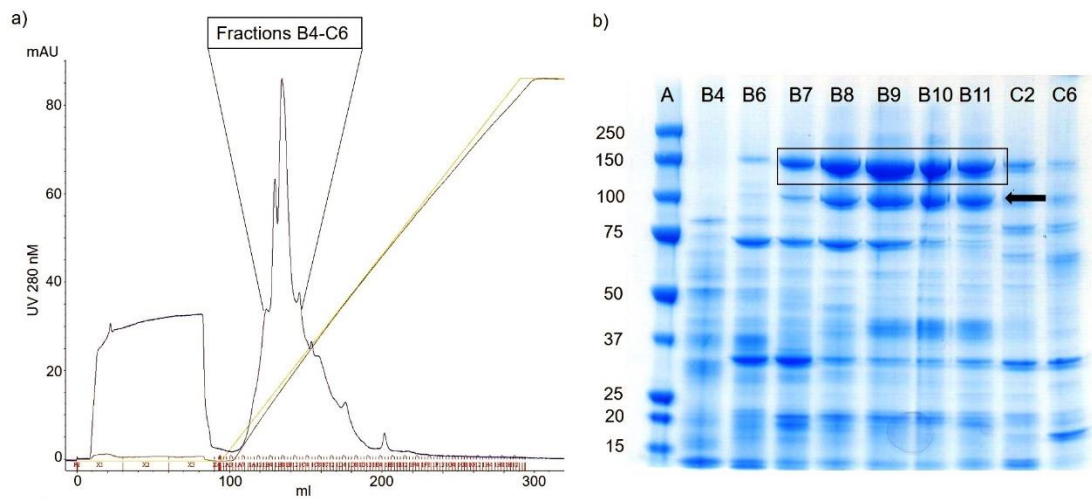


Figure 3.15 Fermentation Three: results of chromatography on Source Q column. a) Chromatogram from a 0.09 - 1 M NaCl gradient applied over 25 column volumes. b) SDS-PAGE run under reducing conditions: the box highlights bands corresponding to rmFH; the arrow indicates bands that probably correspond to clipped rmFH. The bands at ~70 kDa are Endo Hr. All wells were loaded with 20 μ l of sample. A: protein MWt markers.

Fractions B7-B11 (Figure 3.15) were pooled (10 ml). As seen on SDS-PAGE (Figure 3.15 b), the pooled fractions contain lower-molecular weight bands and required further purification. Size-exclusion chromatography was judged to be appropriate here. An aliquot of 0.5 ml of the pooled Source-Q fractions was applied to a Superdex 200 10/300 column; this was repeated several times. Figure 3.16 shows a representative chromatogram and SDS PAGE following one of the elutions.

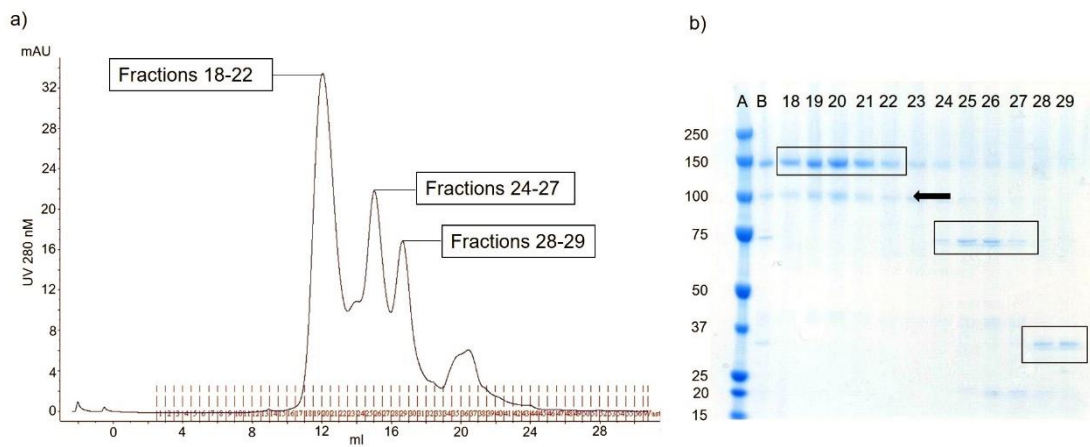


Figure 3.16 Fermentation Three: results of chromatography on 24-ml size exclusion column. a) Chromatogram: elution profile. b) SDS PAGE: run under reducing conditions. B: pre-column sample. Lanes 18-22, rmFH. Arrow: clipped protein. 10 μ l supernatant all wells. A: protein MWt markers.

Size-exclusion chromatography separated rmFH from several other species (fractions 18-22 were pooled), but the band (seen under reducing conditions) at around ~100 kDa, thought to represent 'clipped' protein, remains as would be expected since its MWt will be unaffected. Nonetheless, purification on a larger size-exclusion chromatography column (320-ml Superdex 200 26/600 compared to 24-ml Superdex 200 10/300) was attempted using half of the pooled fractions from the Superdex 200 10/300 run. Figure 3.17 shows the resultant chromatogram and silver staining (reflecting the need for sensitivity) of protein-containing fractions. Note the UV detection range on the chromatogram appears lower than expected; ~ 70 ng of protein was contained in sample G1 which may have been expected to achieve a higher peak UV absorbance.

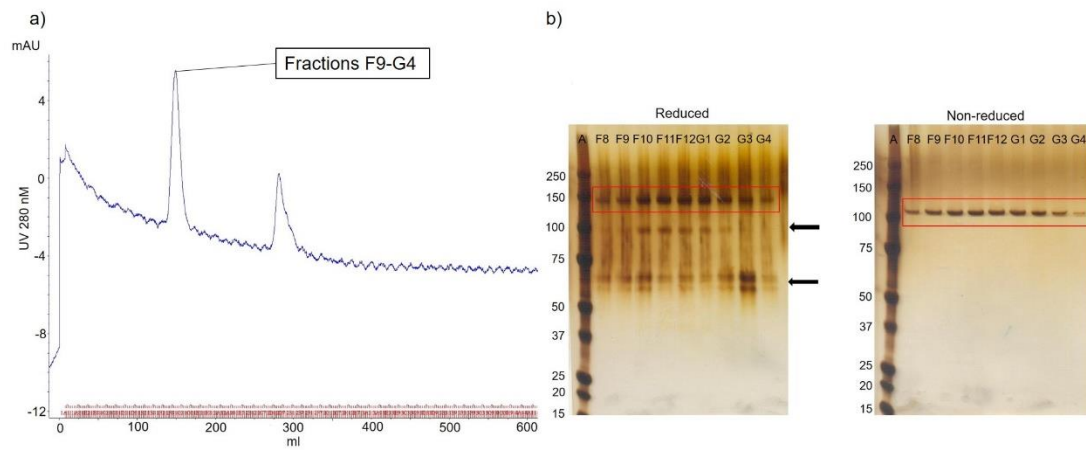


Figure 3.17 Fermentation Three: results of chromatography on a 320-ml size-exclusion column. a) Two main peaks in the chromatogram. b) Silver staining: box shows rmFH. Arrows: clipped protein when reduced. An aliquot of 10 μ l was loaded in each well. A, protein MWt markers.

As can be seen from Figure 3.17 b), the lower-molecular weight protein bands, of which there are clearly two (arrows), remain when rmFH is reduced, reinforcing the likelihood that the bands are indeed clipped portions of the full-length protein.

The total yield of rmFH at the end of the purification process was only 0.27 mg (15 ml at 0.018 mg/ml). The sample was not concentrated further due to the risk of protein loss during the concentration process, and the sample was subsequently used as a positive control in Western blots (Figures 3.20/21/23).

3.1.2.2.4 Fermentation Four

Fermentation Four was a ten-litre fermentation using the same conditions as Fermentation Three, but using a lower pH. It has been reported in the literature that neutral proteases are inhibited if the pH of the fermentation medium is lowered to 3.0 (169, 173, 179), and therefore this method was tried to reduce proteolytic clipping of the secreted protein. During the third and fourth glycerol feeds the pH was set to pH 3.0 and the culture maintained at this pH prior to methanol induction. In total the fermentation received 750 ml methanol, 500 g sorbitol and 150 g tryptone, approximately one third of the amounts received in Fermentation Three. The oxygen demand that reflects methanol metabolism

was slower to increase and took longer to return to baseline after each feed compared to the previous fermentation.

The final volume at the time of harvesting was 11 litres, with 3.5 kg of cells and 7.7 litres of supernatant. The supernatant was noted to be very cloudy and pale green, and remained so post-centrifugation.

Figure 3.19 shows the chromatogram and associated SDS-PAGE following the SP Sepharose cation-exchange step. There is no evidence of significant rmFH production.

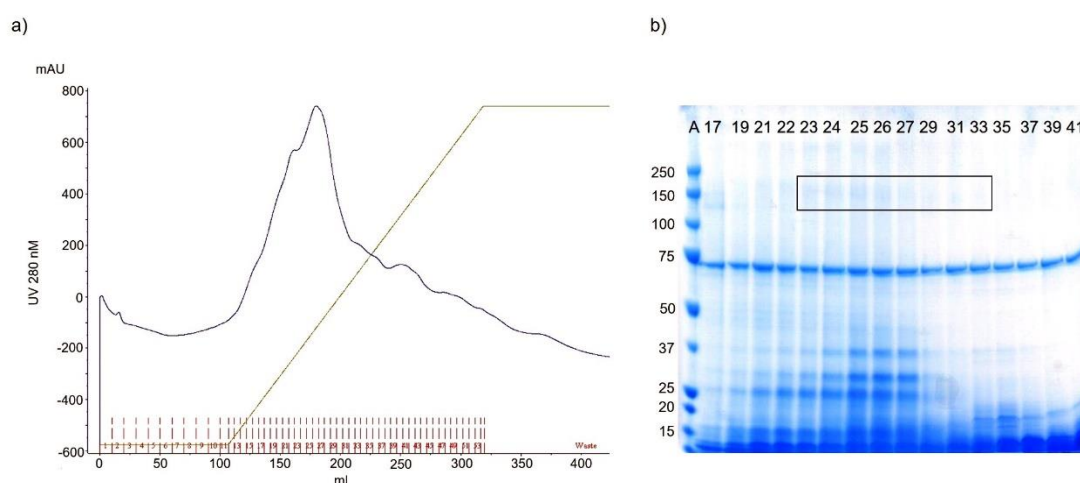


Figure 3.18 Fermentation Four: results of chromatography on SP-Sepharose. a) Chromatogram from a 0.05 M - 1 M NaCl gradient applied over four column volumes. b) SDS PAGE: box shows where band would be expected of rmFH (reduced). Band at ~70 kDa is Endo Hr. All wells were loaded with 10 μ l sample. A: protein MWt markers.

3.1.2.2.5 Fermentation Five

Fermentation Five was a standard ten-litre fermentation, performed under the same conditions as Fermentation Three (which had been the most successful to date in terms of protein production). The only difference was that the Zeocin concentration of the starter culture was 1 mg/ml (as in Fermentation One) as opposed to 500 μ g/ml in Fermentation Three. This was guided by the previously mentioned tentative theory that the higher the concentration of Zeocin a colony tolerates, the higher the copy number of the rmFH gene (166-170).

The fermentation received 1.8 L of methanol (compared to 2.35 L for Fermentation Three) due to slower growth rate of yeast cells as judged by their oxygen consumption. The supernatant was cloudy and green, as noted with previous fermentations. Figure 3.20 shows the chromatogram and SDS-PAGE following the initial SP Sepharose-purification step. Whilst protein bands consistent with rmFH are present, clipped protein is also in evidence. There are more lower-molecular weight bands present compared to Fermentation Three, implying more lysis.

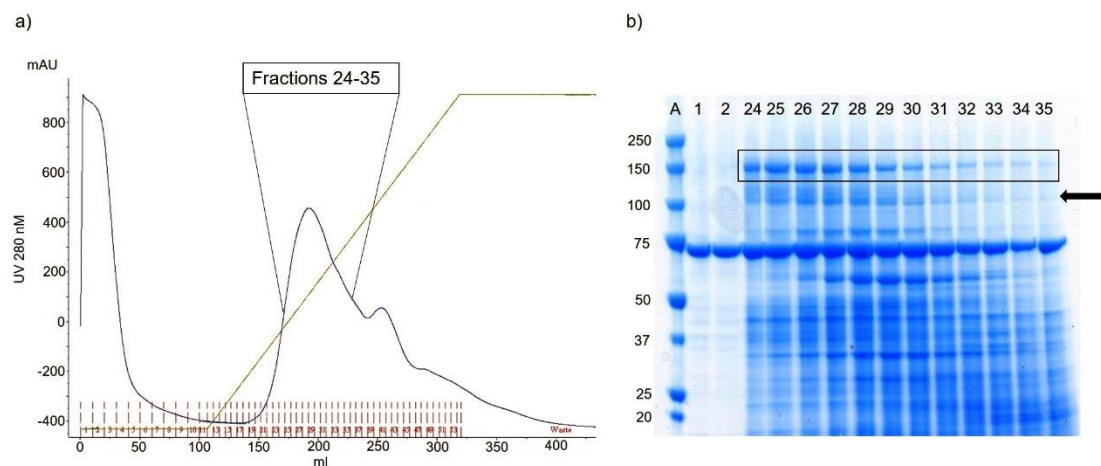


Figure 3.19 Fermentation Five: results of chromatography on SP-Sepharose. a) Chromatogram from a 0.05 M - 1 M NaCl gradient applied over four column volumes. b) SDS PAGE: box shows where band would be expected of rmFH (reduced). Arrow: clipped protein. 10 μ l sample of fraction in each well. Band at ~70 kDa is Endo H_f. A: protein MWt markers.

Fractions containing rmFH were pooled and subjected to further purification by lectin-affinity and anion-exchange chromatographies. As in Fermentation Three, a very low yield of protein was obtained by the end of the purification process, with particular losses at the lectin-affinity chromatography stage due to incomplete deglycosylation.

Therefore, although we now had evidence of rmFH production with pJe912 (as opposed to effectively none with pPICZαB), the amount of purified protein was still 100-fold less than required for any *in vivo* experiments.

3.1.2.3 SacI linearization

3.1.2.3.1 KM71H Test expressions

Updated product information from DNA2.0 suggested that linearization of pJe912 with *SacI* may result in higher protein production levels, compared to linearization with *BamHI*. Whilst the reason for this was not given, presumably linearization with *SacI* resulted in more successful integration events into the KM71H genome. RmFH in pJe912 was subsequently linearized with *SacI* and transformation into KM71H and test expressions were undertaken as previously described.

Figure 3.20 shows Western blots performed after SDS-PAGE on aliquots of the culture medium from small-scale production tests. Note that for these blots, in a departure from the previous studies described above, the primary antibody was sheep anti-human FH diluted 1:200, and the secondary antibody was rabbit anti-sheep IgG diluted 1:20,000 (both from Abcam) (Appendix D).

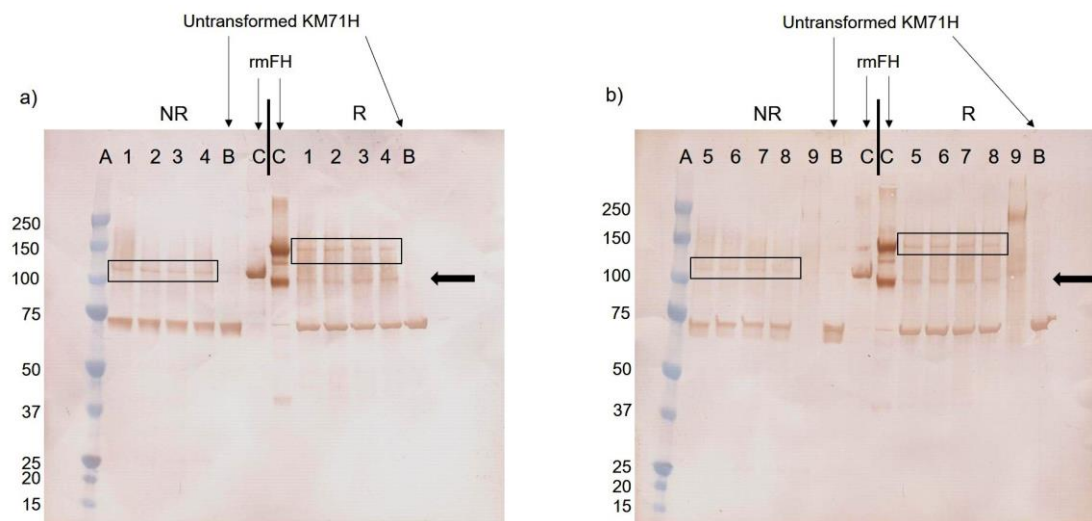


Figure 3.20 Protein production trial of KM71H transformed with *SacI*-linearized plasmid.

Western blots of culture media after spinning out cells. In each lane, an aliquot of 10 μ l was loaded from a total volume of 20 ml. a) Lanes 1-4, four colonies grown on 500 μ g/ml Zeocin, run under NR and R conditions, as indicated. b) Lanes 5-7, three colonies grown on 300 μ g/ml Zeocin; lanes 8-9, two colonies grown on 100 μ g/ml Zeocin run under NR and R conditions, as indicated. Colony 9 was not treated with Endo H_f in error. Boxes highlight the suspected rmFH bands. The large arrow indicates putatively clipped protein. A: protein MWt markers. B: untransformed KM71H cells. C: 0.4 μ M rmFH (from Fermentation Three). The primary antibody was sheep anti-human FH diluted 1:200. The secondary antibody was rabbit anti-sheep IgG diluted 1:20,000 (both from Abcam). The positively stained band – presumably a result of non-specific binding by one or both antibodies – at ~70 kDa is Endo H_f.

The Western blot in Figure 3.20 shows an apparent improvement in rmFH production at the test-expression scale, compared to initial production trials (Figure 3.7 b). This may be due to linearization with *SacI* but could also arise from an improvement in detection as a result of using the alternative antibodies (note these samples had not been subjected to heparin-affinity purification prior to Western blot). Lanes B contain untransformed KM71H as a negative control and show no evidence of rmFH production, as would be expected. The rmFH from Fermentation Three served as a positive control. From a comparison of Figures 3.20a and 3.20b, it appears that colonies grown on 500 µg/ml-Zeocin plates produced more protein than colonies grown on 300 µg/ml-Zeocin plates.

From here onwards all pJe912 linearizations were performed using *SacI* digest.

3.1.3 Recombinant Factor H production with pJe912 in X33

3.1.3.1 Test expressions

X33 is a Mut⁺ strain of yeast, as opposed to KM71H which is a Mut^S strain of yeast. By way of explanation: the *P. pastoris* genome contains two alcohol oxidase genes, *AOX1* and *AOX2*. In Mut⁺ strains both genes are present and functional. In Mut^S strains, *AOX1* has been disrupted and these cells rely on *AOX2* for methanol metabolism, which results in a slower doubling rate (155). As Mut⁺ has a faster growth rate, transformation into X33 was performed to ascertain whether protein production levels could be improved by using this strain. Figure 3.21 shows the Western blot obtained from culture medium following small-scale test protein production.

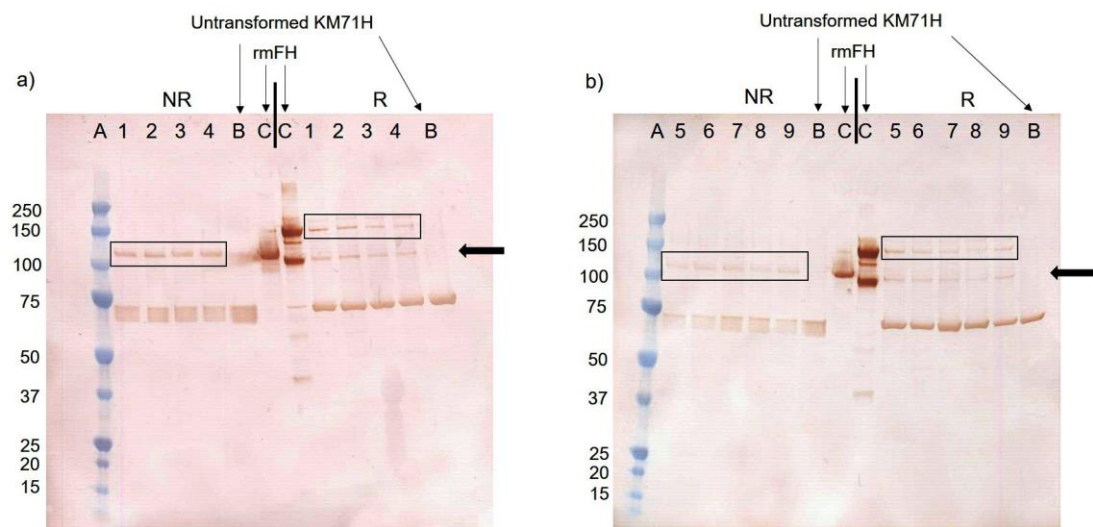


Figure 3.21 Protein production trial for X33 *P. pastoris*. Western blots of culture media after spinning out cells. In each lane, an aliquot of 10 μ l was loaded from a total volume of 20 ml a) Lanes 1-4, 500 μ g/ml Zeocin colonies, NR/R b) Lanes 5-7, 300 μ g/ml Zeocin colonies, Lanes 8-9 100 μ g/ml Zeocin colonies, NR/R. Boxes show rmFH. Arrow: clipped protein. A: protein MWt markers. B: untransformed KM71H. C: 0.4 μ M rmFH (from Fermentation Three). Antibodies as in Figure 3.20. The band at ~70 kDa is Endo H_i.

Figure 3.21 shows successful production of rmFH in the X33 strain of *P. pastoris* but the yield appears no higher than from KM71H cells. As in Figure 3.20, colonies grown on 500 μ g/ml Zeocin appear to produce more protein than lower Zeocin-concentration colonies. Clipped rmFH was present in all samples.

3.1.3.2 Fermentation of X33 cells

For side-by-side comparison purposes, a ten-litre fermentation should have been undertaken with *SacI*-linearized pJe912 containing the DNA for rmFH in both KM71H and X33 strains of *P. pastoris*. Due to equipment-availability issues however, this was not possible. Instead X33 alone was selected for a trial, based on the positive appearance of Western blots for test-expression. Moreover the X33 strain had not been used previously in the lab and so was of novel interest for rmFH production. The starter culture was grown in media containing 500 μ g/ml Zeocin.

During fermentation, methanol was added according to the feeding status of the yeast as before. On average two 150-ml methanol feeds were provided per

day, over the four-day methanol-feeding period (as opposed to four-six 200-ml feeds per day in the previous ten-litre fermentations). Therefore only 1.6 L of methanol was added in total, compared to 2.35 L for Fermentation Three. This implied longer duplication times of the yeast cells, which was not expected.

Cells were spun down and supernatant harvested as before. This was then passed over a SP-Sepharose column in an initial purification step, as described above. The outcome is shown in Figure 3.22.

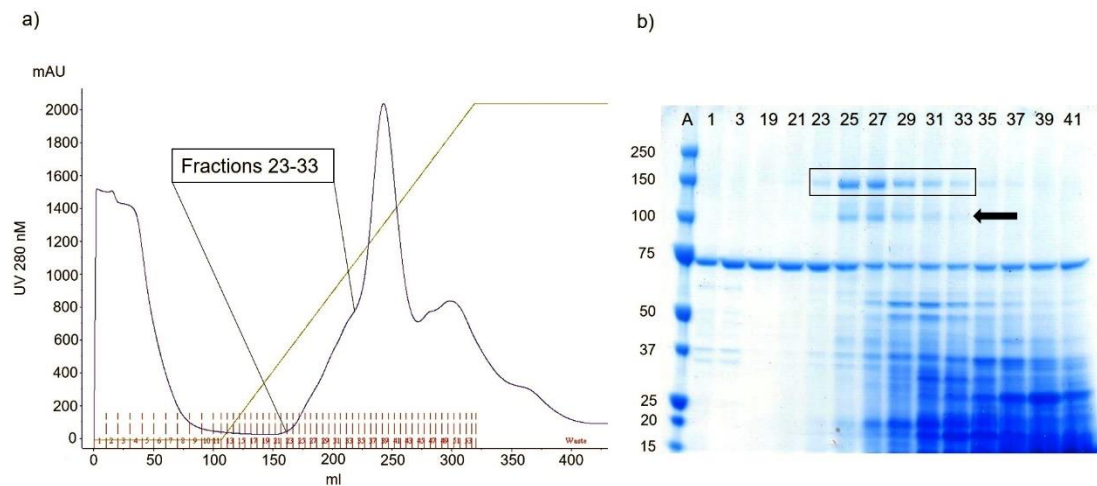


Figure 3.22 X33 Fermentation: results of chromatography on SP-Sepharose. a) Chromatogram from a 0.05 M - 1 M NaCl gradient applied over four column volumes. b) SDS PAGE: box shows where a band would be expected for rmFH (reduced). Arrow: clipped protein. The band at ~70 kDa is Endo H_f. In each well is a 10 µl sample. A: protein MWt markers.

Figure 3.22 b) shows the presence of protein bands consistent with rmFH, but the yield was disappointing given the promising results of small-scale production trials. A significant quantity of lower-molecular weight proteins, probably originating from lysed cells, was eluted at a higher salt concentration than rmFH (fractions 29 onwards) as also seen in Figures 3.10b), 3.12b), 3.13b), and 3.19b).

3.1.4 Recombinant murine Factor H production with pJe912 in SMD1168H

Proteolytic cleavage reduces the yield of recombinant proteins but this may be acceptable provided proteolytic products are resolvable. In disulfide-rich

proteins, however, cleavage may occur within the sequence between two cysteine residues participating in a disulfide bond. In this case, the term “clipped” may be used since the two products remain disulfide linked to each other under non-reducing conditions and are hard to resolve from unclipped protein by chromatography. Notably, CCP modules have two disulphide bonds in a 1-3, 2-4 pattern, with cysteine 1 very close to the N terminus of the module and cysteine 4 very close to the C terminus. Hence, when FH is cleaved within a ~60 residues CCP module (as opposed to within the 3-8 residue linkers between the fourth cysteine of one modules and the first cysteine of the next) it is clipped. Evidence for clipping – the appearance of lower-molecular weight bands in SDS-PAGE only when samples are run under reducing conditions - was detected in all preparations of recombinant murine FH described so far.

In an attempt to reduce proteolysis, rmFH in pJe912 was transformed into a Protease A-deficient, Mut⁺, strain of *P. pastoris*, SMD1168H.



Figure 3.23 RmFH in SMD1168H trial. Western blot of culture media after spinning out cells. Lanes 1-6 show 10 µl samples from 300 µg/ml Zeocin colonies. B: 5 µl of 0.4 µM rmFH (from Fermentation Three). Boxes indicate expected position of bands corresponding to rmFH. Arrow: clipped protein. Band at ~70 kDa is Endo H_r. Antibodies as Figure 3.20. R = reducing; NR = non-reducing conditions. A: protein MWt markers.

Figure 3.23 shows the Western blot from a successful transformation and subsequent test expressions. No marked improvement in protein expression

is apparent and evidence is still present of clipped protein. Protein production in this strain of *P. pastoris* was therefore not pursued further.

3.1.5 Recombinant murine Factor H production with Protein Disulfide Isomerase

Multiple-milligram quantities of rmFH are required to undertake the planned experiments in CFH^{-/-} mice. Despite using two different codon-optimised genes and three strains of *P. pastoris*, along with attempts to optimise production conditions, only very small quantities of rmFH were obtained that were inadequate for *in vivo* studies. It was vital to address this issue of low yield before moving on to the next phase of the project.

An established way to improve recombinant protein production is to increase the gene copy number (166-170) but this proved unhelpful in the case of rmFH. The pJe912 vector carrying the rmFH gene contains a Zeocin-resistance selectable marker. Transformed colonies able to grow at higher Zeocin concentrations are likely to possess multiple copies of the resistance gene and therefore to have additional copies of the rmFH gene also. If transcription is limiting, this should boost protein production. But in the rmFH-production attempts described above, colonies with higher resistance to Zeocin produced less rather than more protein (e.g. Fermentation Five grown in 1mg/ml Zeocin versus Fermentation Three grown in 0.5 mg/ml Zeocin). From this it may be inferred that some other factor is acting as a bottleneck on production. It was also noted that growth in the fast-growing Mut⁺ strain X33 produced less protein than in the slower growing Mut^s (KM71H) strain. In general lower rates of cell division, transcription and translation are thought to be preferred for protein production in those cases where folding is the rate-limiting step (180).

Secretory proteins such as rmFH are targeted to the endoplasmic reticulum (ER) where, with the help of ER-resident proteins, they undergo folding and post-translational modifications that include the first step of N-glycosylation and the formation of disulfides. Misfolded proteins remain in the ER for further attempts at folding or can be removed *via* ER-associated degradation (181, 182). It was speculated that production of a protein like rmFH with forty

disulphide bonds in 20 sequential modules might overwhelm the disulfide formation machinery within the endoplasmic reticulum of the cell. This effect would be exacerbated by a high gene-copy number (183, 184).

The ER-associated chaperone protein disulfide isomerase (PDI) assists in the formation of disulfide bonds (185). This is especially important where there are multiple possible patterns of disulfide bonds but only one of these patterns is consistent with the native 3D structure of the functional protein. In the literature over-expression of PDI was shown, in several instances, to increase recombinant protein production by two or three-fold, but in one case by as much as sevenfold (186-193). In an attempt to improve the production of rmFH, a strain of *P. pastoris* designed to overproduce *P. pastoris* PDI was developed. Once expression of the *PDI* gene had been confirmed, this strain could be transformed with rmFH DNA for protein-production trials.

3.1.5.1 Protein Disulphide Isomerase Expression

An optimised gene for *P. pastoris* PDI (DNA 2.0), in the vector pPIC3.5K and under control of the *AOX1* promoter, was transformed into *P. pastoris* KM71 cells and the suspension plated onto minimal dextrose (MD) agar plates. KM71 (unlike KM71H) has a mutation in the *HIS4* gene (*HIS4*⁻). Hence it requires the use of vectors containing *HIS4*, such as pPIC3.5K, to grow in media lacking histidine. Successfully transformed clones therefore have the ability to grow in minimal dextrose (MD) (with no histidine).

Small-scale trials of protein production were performed as before. PDI is an intracellular protein, hence the cell pellet as well as the supernatant were retained post-harvest. Protein production was assessed by SDS-PAGE (Figure 3.24) of both lysed cells and growth media.

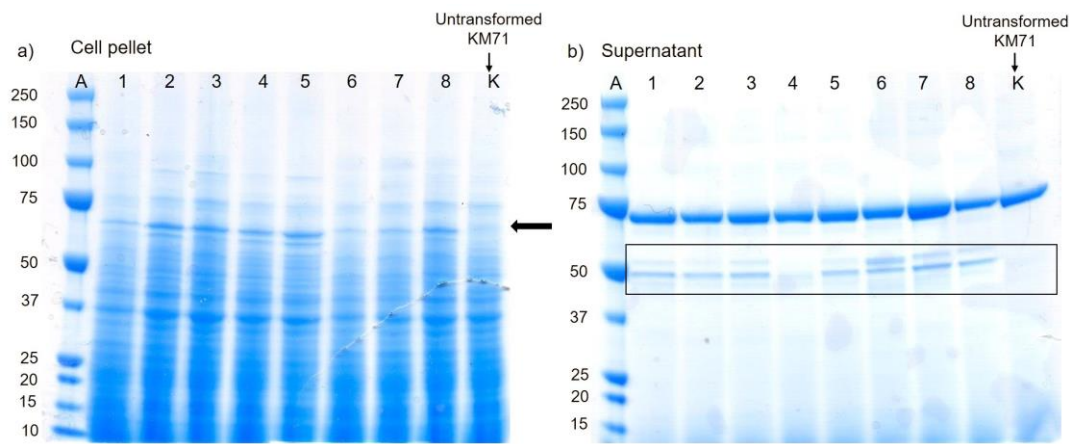


Figure 3.24 PDI production trials. a) SDS PAGE analysis of the pellet following cell lysis and spinning down. Lanes 1-8, colonies. K: untransformed KM71. Lysates were diluted 1:10 and reduced before loading on to the gel. Arrow: possible PDI b) SDS PAGE, analysis of the supernatant. Lanes 1-8, colonies. K: untransformed KM71. Box: possible PDI. Band at ~70 kDa is Endo H α . Each lane was loaded with 20 μ l of protein solution. A: protein MWt markers.

The predicted molecular weight of *P. pastoris* PDI is ~58 kDa. Coomassie-stained bands consistent with proteins of 50-60 kDa were visible in the SDS-PAGE of test production growth media (*i.e.* the secreted protein fraction) (Figure 3.24b) lanes 1-8). No equivalent band was detectable in the media from non-transformed KM71 cells (lane K). These bands are candidates for PDI although PDI should not be secreted into the media.

Proteins solubilised in SDS from the lysed cell pellet were diluted 1:10 for visualisation on the SDS-PAGE gel (Figure 3.24a). A protein band is present at 60-70 kDa (lanes 1-8). No equivalent band was present in the cell pellet of untransformed KM71, so this is also a candidate for PDI. Assuming this to be the case, Colony 2 (see Figure 3.26) was chosen for further transformation with rmFH in pJe912.

3.1.5.2 Trial productions of recombinant murine Factor H in PDI-KM71H cells

Figure 3.25 shows a Western blot of the small-scale trial production of rmFH by pJe912-transformed PDI-KM71 cells.

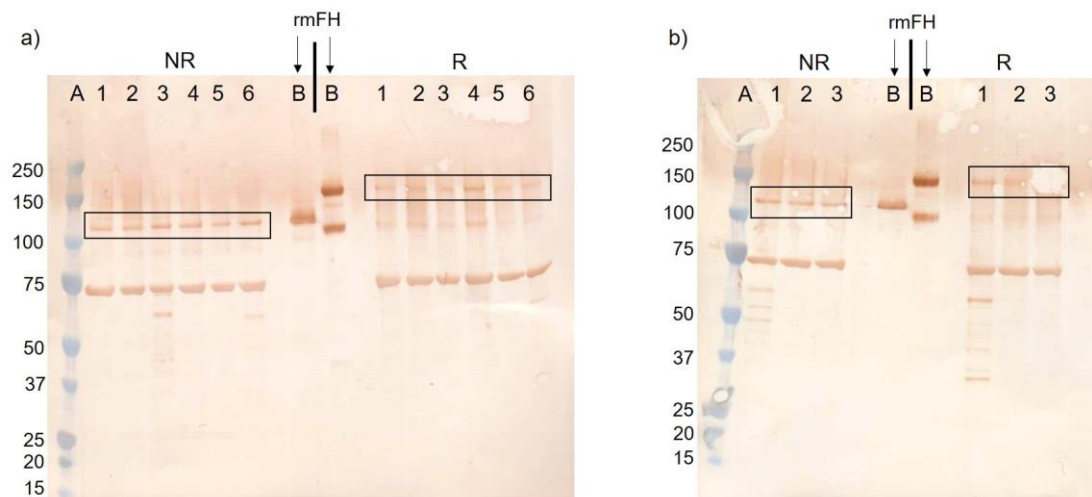


Figure 3.25 Western blot for rmFH produced by pJe912-transformed PDI-KM71 cells. a) Lanes 1-2, cultures grown on 100 µg/ml Zeocin plates. Lanes 3-6, cultures grown on 300 µg/ml Zeocin plates b) Lanes 1-3, cultures grown on 500 µg/ml Zeocin plates. B: 5 µl 0.4 µM rmFH (from Fermentation Three). R = reducing conditions, NR = non-reducing conditions. Box: expected position of rmFH bands. The band at ~70 kDa is Endo H. Antibodies as in Figure 3.20. An aliquot of 10 µl supernatant was loaded into each well. A: protein MWt markers.

The Western blots suggest rmFH has been produced, although when run under reducing conditions proteolytic clipping of rmFH remains evident. While these Western blots are not entirely quantitative, expression is vastly improved compared to that of pJe912 in KM71H *i.e.* without PDI co-expression (Figure 3.20). Ideally these blots should have included a direct comparison of rmFH production in KM71(H) with and without PDI.

Figure 3.26 summarises the results of an ELISA performed (by Dr Eliza Makou, protocol in Appendix E) to compare the production of recombinant *human* FH (162-rhFH) by KM71H versus the PDI-KM71 strain described above. This ELISA utilised the very high affinity of human FH for the bacterial protein domain PspCN that was used to capture rhFH (160). PspCN does not bind to murine FH directly and hence this method was not used for quantifying rmFH production. Nonetheless it suggests at least a ten-fold improvement for rhFH production. The difference in the case of rmFH, that expressed only very sparingly from KM71H cells, is clearly even greater.

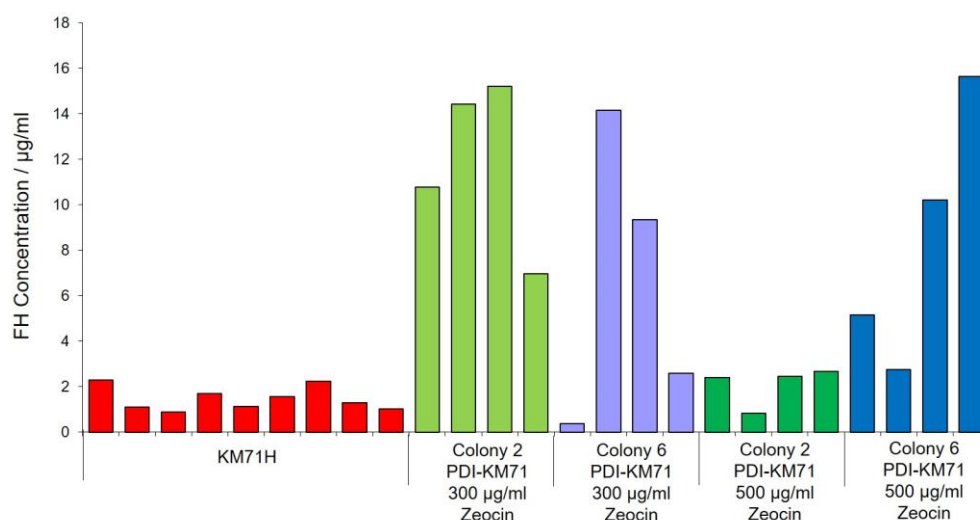


Figure 3.26 ELISA of I62-rhFH production in KM71H and PDI-KM71. A comparison between rhFH production in KM71H (no PDI) compared to colonies 2 and 6 from Figure 3.24, grown on either 300 µg/ml or 500 µg/ml Zeocin plates and expressed in small-scale shaker flasks.

While it is clear from Figure 3.26 that KM71H produces generally much less rhFH than PDI-KM71, comparison of the amounts of rhFH produced by PDI-KM71 grown on 300 µg/ml Zeocin plates and 500 µg/ml Zeocin plates suggests that using a colony grown on a *lower* zeocin-concentration plate appears to produce more rhFH, (particularly in the case of colony 2). Thus despite the co-expression of PDI, FH production may remain limited by disulfide production. Colony 2 (as in 3.1.5.2) PDI-KM71 was used as the base strain for all further work with rmFH as below.

3.1.5.3 Fermentations

Based on the results obtained for Figure 3.26, a 300-µg/ml Zeocin plate-grown colony, then a 300-µg/ml starter culture, was used to inoculate a series of PDI-KM71 fermentations.

All fermentations were on a ten-litre scale, with a four-day methanol-induction period, and taking on board lessons learnt from the above-described production attempts. Standard methanol feeds were replaced with a 4:1 methanol/glycerol feed (194-196). Mixed feeding is thought to allow for a higher cell density due to continued growth with glycerol during the methanol

induction phase. Sorbitol was not added due to the concern that a too rapid increase in cell mass might contribute to protein misfolding and cell lysis. Tryptone was added as previously.

Fermentation	Zeocin concentration of inoculation culture ($\mu\text{g/ml}$)	Methanol/glycerol feed	Protease Inhibitor
1	300	Batch-fed	PMSF
2	300	Pump-fed	PMSF
3	300	Pump-fed	Pefabloc
4	300	Pump-fed	Anhydrous PMSF

Table 3.2 Summary of rmFH pJe912 in PDI-KM71 fermentation conditions

Fermentations One and Two were run concurrently as were Fermentations Three and Four. Because a maximum of two fermentations could be run at the same time it was decided to compare batch-feeding and pump-feeding. It had been proposed that continuous delivery of glycerol and methanol using a pump would reduce the possibility of under- or oversupply and hence increase protein production (196).

3.1.5.3.1 Fermentation One (Batch-fed and with PMSF added)

This fermentation received approximately 800 ml methanol/glycerol feed per day. At the time of harvesting the supernatant was brown in colour, compared to the green colour of most fermentations observed previously.

Daily samples were taken for analysis by SDS PAGE during the fermentation period. (Figure 3.27).

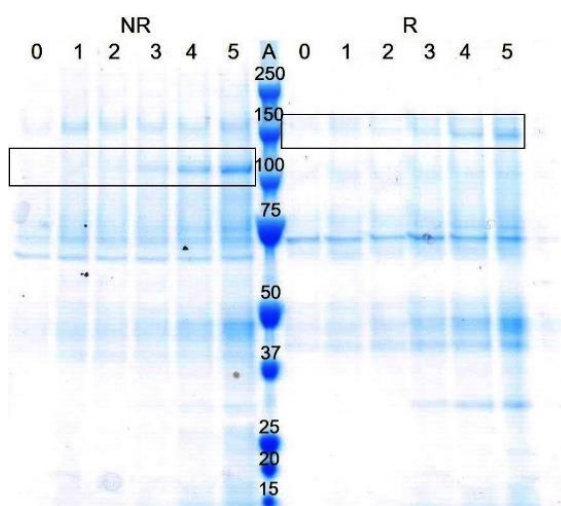


Figure 3.27 SDS PAGE of daily samples from Fermentation One (batch-fed, PMSF added). Lanes 0-5, days of fermentation. 0, glycerol feed. 1-4, methanol/glycerol feed days. 5, harvest. NR = non-reducing conditions R = reducing conditions. Box: rmFH. The band at ~70 kDa is Endo H_f. All wells were loaded with 10 µl supernatant. A: protein MWt markers.

Figure 3.27 shows that by the fourth day of methanol/glycerol feed, there is clear evidence of rmFH production. This was the first time that rmFH was detected by Coomassie blue stained SDS-PAGE in crude supernatant from the fermenter, prior to any purification or enrichment. Adding a fourth day of methanol feeding prior to harvest was advantageous in terms of protein production. Direct visualization of the SDS-PAGE gel suggested some proteolytic clipping had occurred.

Instead of using SP-Sepharose for cation-exchange chromatography it was decided to use Toyopearl SP-650 resin (159). This decision was based on the experience of other laboratory members who had found this resin was advantageous for separating recombinant human FH from *P. pastoris* proteins (personal communication, Dr Andrew Herbert). Like SP-Sepharose, Toyopearl SP-650 is a strong cation-exchange resin. Figure 3.28 shows the resulting chromatogram and SDS-PAGE.

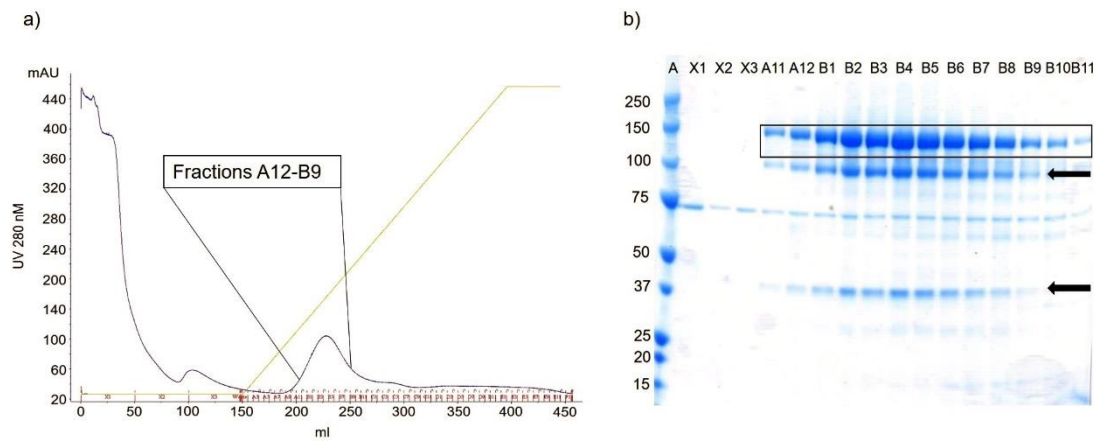


Figure 3.28 RmFH PDI-KM71 Fermentation One after chromatography on Toyopearl SP-650. a) Chromatogram from a 0.05 - 0.5 M NaCl gradient applied over four column volumes. b) SDS-PAGE (under reducing conditions). The box shows where a band would be expected for rmFH (reduced). Arrow: suspected clipped rmFH. The band at ~70 kDa is Endo Hr. All wells were loaded with 10 μ l samples. A: protein MWt markers.

In comparison to the cation-exchange step of the most successful of the fermentations performed prior to co-expression with *PDI* (Figure 3.13 b), the SDS-PAGE gel reveals a much higher ratio of (candidate) rmFH to other species (presumed to be *P. pastoris* proteins). Evidence of proteolytic clipping remains.

The estimated protein concentration of the central peak fraction by spectroscopy was 0.5 mg/ml (in 5-ml fractions). Fractions A12 to B9 were pooled to give a volume of 50 ml (~ 21 mg of protein in total). This was treated with Endo Hr, and subsequently applied to a column containing lectin-affinity resin (Figure 3.29).

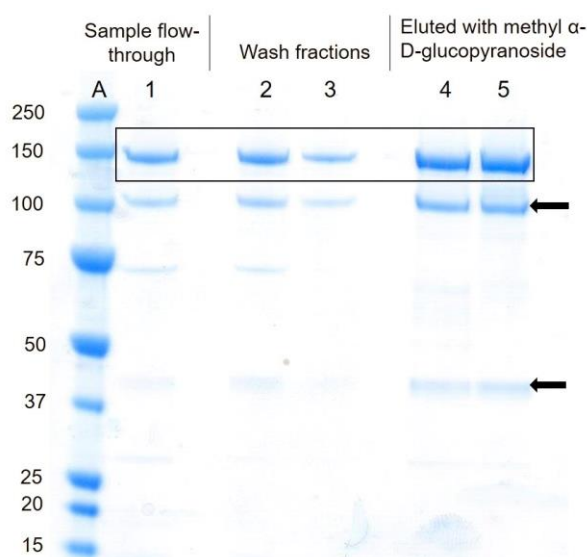


Figure 3.29 RmFH PDI-KM71 Fermentation One after lectin-affinity chromatography. SDS PAGE (run under reducing conditions) . Lane 1, flow-through; lanes 2-3, wash fractions; lanes 4-5, fractions eluted with 1 M methyl α -D-glucopyranoside. Aliquots of 20 μ l were loaded in all wells. The box shows where a band would be expected for rmFH (reduced). Arrow: clipped protein. A: protein MWt markers.

Lanes 4-5 in Figure 3.29 show that a significant proportion of rmFH remained attached to the lectin affinity column suggesting it was not fully deglycosylated. This phenomenon was noted previously (Figure 3.14) but it was not expected in the present case given that more Endo H_f was used to treat rmFH compared to the amount used to successfully treat rhFH (~ 150,000 units of Endo H_f for rmFH at 37 °C for 1 hour; ~ 30,000 units of Endo H_f at 37 °C for 2-3 hours for rhFH (23). A test incubation with Endo H_f at 37 °C was conducted on samples recovered from the cation-exchange step used previously. Incubation periods ranging from 1 – 8 hours did not deliver any further deglycosylation (data not shown). A more exhaustive comparison, entailing lectin-affinity chromatography and elution with 1 M methyl α -D-glucopyranoside was not performed at this stage. It was decided, for future work, to use an excess of Endo H_f and a shorter, one-hour, incubation period at 37 °C.

Fractions 1-3 from Figure 3.29 were pooled with similar fractions from Fermentation Two (below) and buffer-exchanged into the equilibration buffer

for loading on to an anion-exchange (Source-Q) column chromatographic step (Figure 3.33).

3.1.5.3.2 Fermentation Two (Pump-fed and with PMSF added)

In this fermentation, approximately 1700 ml methanol/glycerol were supplied each day *via* a peristaltic pump (twice the volume batch-fed to Fermentation One). At the time of harvesting the supernatant was brown in colour.

Daily samples were taken during the fermentation period and analysed by SDS PAGE (Figure 3.30).

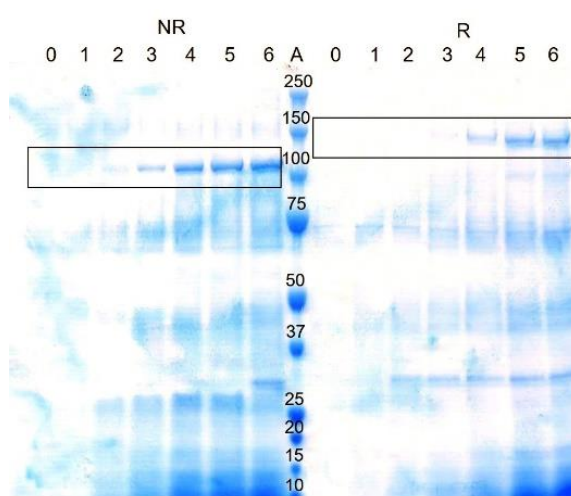


Figure 3.30 SDS-PAGE of daily samples from Fermentation Two (pump-fed, PMSF added). Lanes 0-5, days of fermentation. 0, glycerol feed. 1-4, methanol/glycerol feed days. 5, harvest. 6, harvested supernatant with PMSF added prior to the deglycosylation step. NR = non-reducing conditions, R = reducing conditions. Aliquots of 10 μ l were loaded into all wells. A: protein MWt markers.

Figure 3.30 suggests that using the pump-fed method of methanol/glycerol delivery results in a larger amount of rmFH being produced compared to the batch-fed method (Figure 3.27), although a direct comparison is not shown here.

Figure 3.31 shows the chromatogram and SDS-PAGE following the cation-exchange step using Toyopearl SP-650 resin. The peak absorbance at 280 nm is ~300 mAU (peak 350 mAU, baseline 50 mAU), compared to ~80 mAU for Fermentation One. Note that only 3 μ l of sample (rather than 10 μ l as used

routinely) was loaded on SDS-PAGE for fractions corresponding to the elution peak.

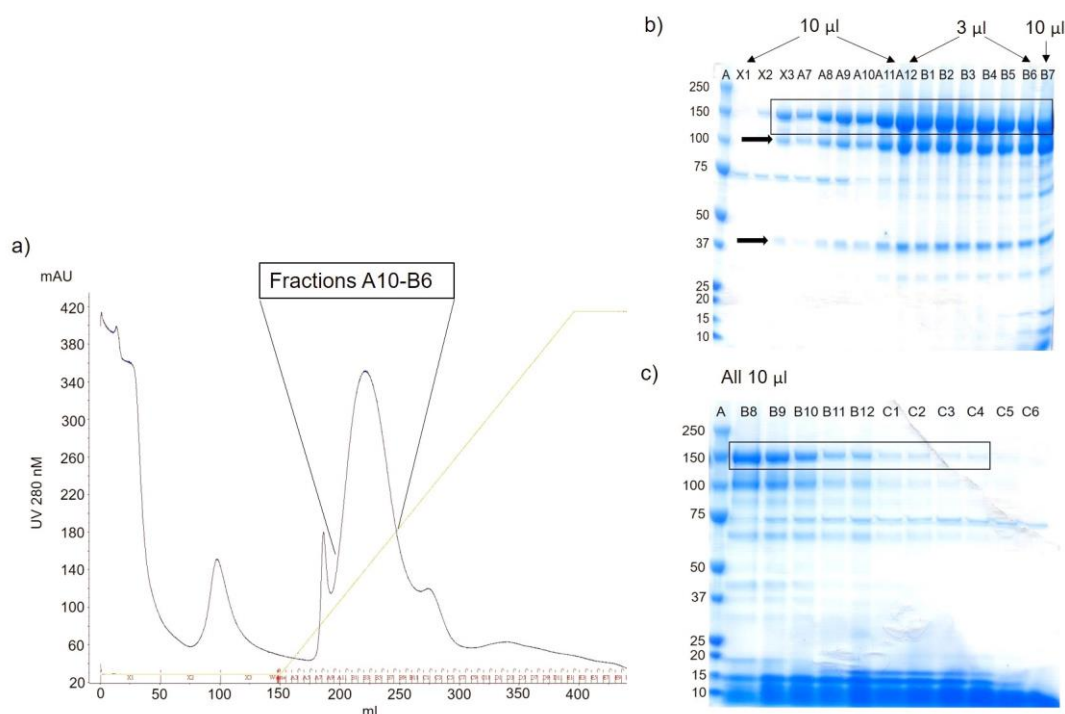


Figure 3.31 RmFH PDI-KM71 Fermentation Two after chromatography on Toyopearl SP-650. a) Chromatogram from a 0.05 M - 0.5 M NaCl gradient applied over four column volumes. b) and c) SDS-PAGE (run under reducing conditions) Box shows where a band would be expected for rmFH (reduced). Arrow: clipped protein. The band at ~70 kDa is Endo H_f. Volumes loaded into wells are indicated. A: protein MWt markers.

The estimated protein concentration (2.3.8) of the central peak fraction by spectroscopy was 3 mg/ml (5 ml fraction). It was found that once deglycosylated the protein precipitated out of solution at concentrations > 2 mg/ml, and hence required dilution by 1:2, or 1:3, with 20 mM potassium phosphate, pH 6, 200 mM NaCl, prior to further purification. A standard practice implemented between protein purification steps is to keep the samples on ice (reduced protease action at lower temperatures), but it was also noted that cold increased the likelihood of precipitation of rmFH. Hence, the lectin-affinity column and further buffer-exchange steps in this and subsequent purifications were performed at room temperature.

Fractions A10 to B6 were pooled to give a volume of 105 ml (~ 96 mg of protein in total, an almost five-fold increase compared to Fermentation One at this point). This was deglycosylated then applied to the lectin-affinity chromatography column (Figure 3.32).

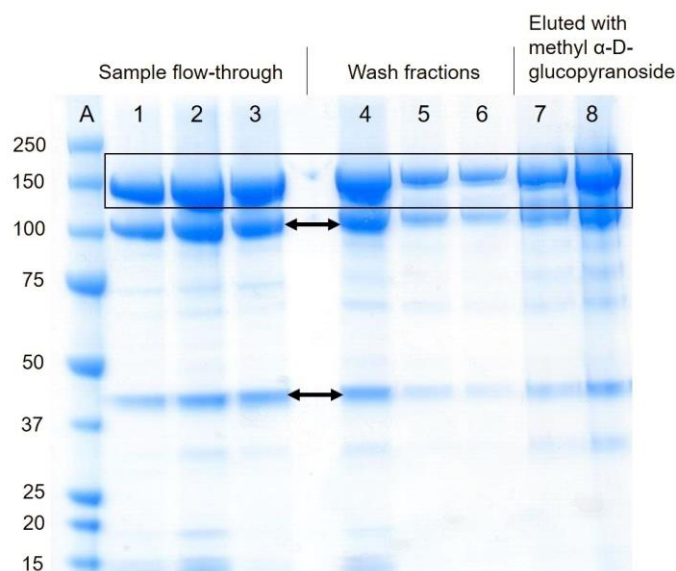


Figure 3.32 RmFH PDI-KM71 Fermentation Two after lectin-affinity chromatography. SDS PAGE under non-reducing conditions. Lane 1-3, sample flow-through; lanes 4-6, wash fractions; lanes 7-8, fractions with 1 M methyl α -D-glucopyranoside. Aliquots of 20 μ l supernatant were loaded in all wells. Box shows where rmFH band would be expected. Arrow: clipped protein. A: protein MWt markers.

Lanes 7-8 in Figure 3.32 show that whilst a proportion of the rmFH has remained attached to the lectin-affinity column (eluted with 1 M methyl α -D-glucopyranoside) it is a less significant proportion of the overall protein sample compared to the previous fermentation (Figure 3.29).

Fractions 1-5 were pooled (125 ml) and buffer-exchanged into the equilibration buffer and combined with material from Fermentation One for the ensuing anion-exchange (Source Q) column chromatographic step. Due to the large volume (135 ml) of the combined sample, it was divided in two and each sample dialysed separately in 4 L of Source Q equilibration buffer, twice.

Three Source Q runs were performed due to the sample volume. Figure 3.33 shows a representative chromatogram with SDS-PAGE.

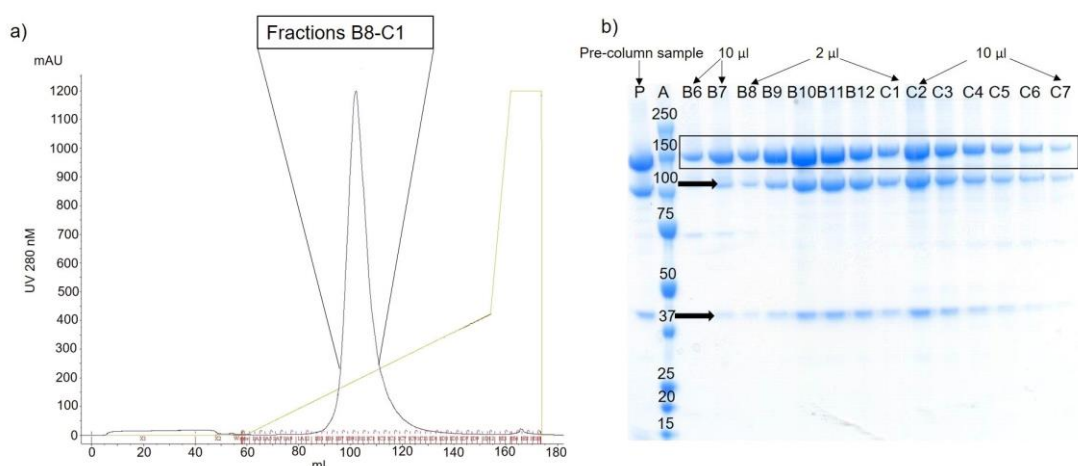


Figure 3.33 RmFH PDI-KM71 combined Fermentation One and Two after chromatography on Source Q. a) Chromatogram from a 0.09 - 1 M NaCl gradient applied over 12 column volumes. b) SDS PAGE run under reducing condition. Box shows where a band would be expected for rmFH. Arrow: clipped protein. P: pre-column sample, 10 μ l. B6-B7, C2-C7: 10 μ l from each fraction. B8-C1: 2 μ l from each fraction. A, protein MWt markers.

The fractions corresponding to the centre of the peak appeared to be virtually free of contaminants (aside from the two polypeptides thought to correspond to “clipped” protein products). Compared with the equivalent gel (Figure 3.15) obtained without PDI co-expression, the very marked improvement in the ratio of rmFH to *P. pastoris* proteins reflects the much greater yield of the target protein as well as reduced cell lysis.

Fractions B8-C1 were pooled from each Source Q run. The total yield of rmFH combined from these two fermentations of PDI-KM71 was 31 mg (*i.e.* 36 ml of 0.86 mg/ml). This was about two orders of magnitude higher than what had been obtained with KM71H although a direct comparison is difficult. The combined yield of protein following the cation-exchange purification step for Fermentations One and Two was ~ 117 mg, therefore a pooled final yield of 31 mg is a combined loss of ~ 75% of protein during the deglycosylation, lectin-affinity and anion-exchange steps.

3.1.5.3.3 Fermentation Three, pump-fed Pefabloc

This fermentation used a peristaltic pump to feed methanol and glycerol, as in Fermentation Two. In place of PMSF, 250 mg of the protease inhibitor Pefabloc

SC (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, AEBSF, Sigma Aldrich) was added every 24 hours during the induction phase. Like PMSF, Pefabloc is a serine protease inhibitor, but it has the advantage of being non-toxic (PMSF is a neurotoxin) and stable in aqueous buffer solutions. Moreover, PMSF has limited solubility and is unstable in aqueous solutions (stock solutions are made in anhydrous isopropanol). There was therefore concern that it only remained active for a short time. Other group members (personal communication Dr Andrew Herbert, Dr Nicola McIntosh) had found the use of Pefabloc in recombinant protein production to be superior to PMSF in terms of reduction of clipped protein.

At the time of harvesting, it was obvious that there was an increased biomass of cells compared to previous fermentations. Indeed, the harvested supernatant required an additional 20 minutes of spinning in the centrifuge to adequately pellet the cells and thus enable the supernatant to be decanted.

Figure 3.34 shows the SDS PAGE of supernatant from daily samples during the fermentation process.

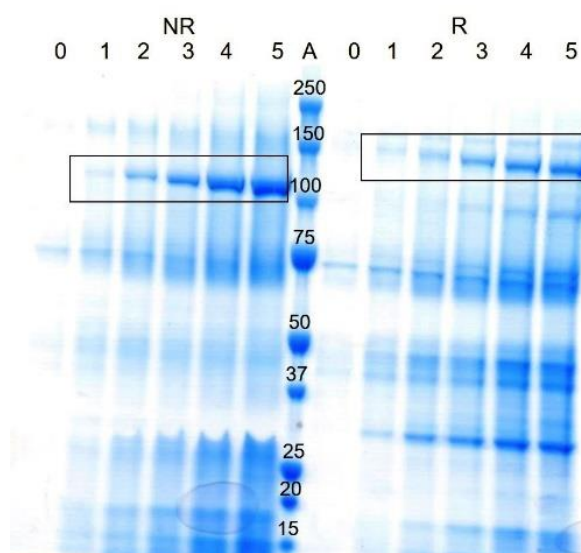


Figure 3.34 SDS-PAGE of daily samples from Fermentation Three (pump-fed, Pefabloc added). Lanes 0-5, days of fermentation. 0, glycerol feed. 1-4, methanol/glycerol feed days. 5, harvest. NR = non-reducing conditions, R = reducing conditions. Aliquots of 10 μ l were loaded into all wells. A: protein MWt markers.

Comparing Figure 3.30 and Figure 3.34 appears to show that the use of Pefabloc instead of PMSF may result in increased rmFH production.

Figure 3.35 shows the chromatogram and SDS-PAGE following the cation-exchange step using Toyopearl SP-650 resin. The peak absorbance at 280 nm is ~380 mAU (peak 400 mAU, baseline 20 mAU), in comparison to ~350 mAU for the equivalent chromatogram for Fermentation Two.

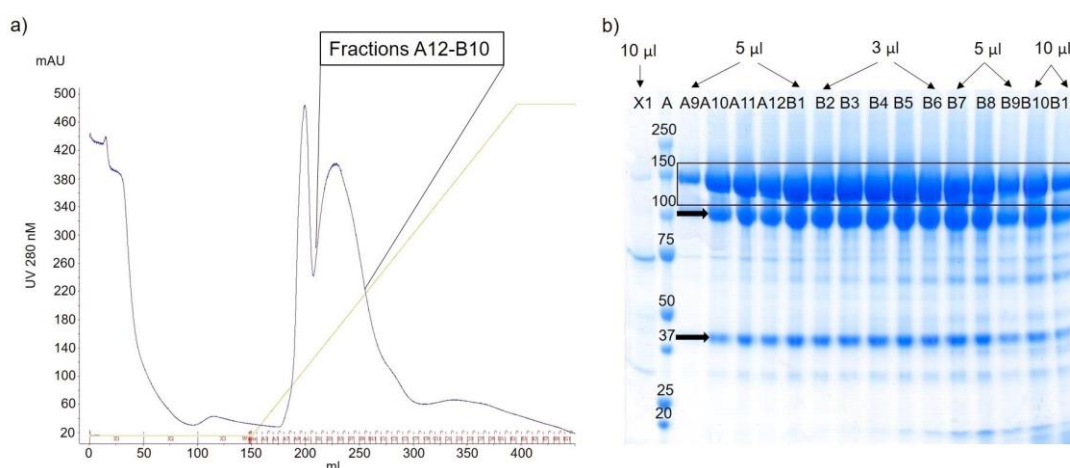


Figure 3.35 RmFH PDI-KM71 Fermentation Three after chromatography on Toyopearl SP-650. a) Chromatogram from a 0.05 - 0.5 M NaCl gradient applied over four column volumes. b) SDS PAGE. Box shows where band would be expected of rmFH (reduced). Arrow: clipped protein. X1, B10-11: 10 μ l supernatant. A9-B1, B7-9: 5 μ l of supernatant. B2-B6: 3 μ l of supernatant. A: protein MWt markers.

The protein concentration of the central peak fraction by spectroscopy was 4.5 mg/ml (5 ml fraction) of rmFH. Fractions with an estimated concentration > 2 mg/ml required dilution by 1:2 or 1:3 (20 mM potassium phosphate, pH 6, 200 mM NaCl) to avoid precipitation prior to further purification (see above). Evidence of protein clipping is still visible on the SDS-PAGE gel despite the use of this alternative protease inhibitor (arrows, Figure 3.35 b). Fractions A12-B10 were pooled to give a total volume of 130 ml (~ 166 mg of protein), which was deglycosylated and applied to the lectin-affinity column (Figure 3.36).

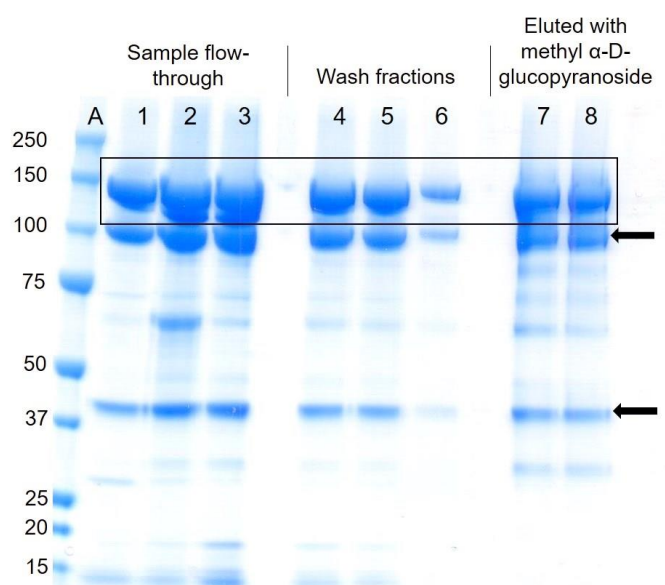


Figure 3.36 RmFH PDI-KM71 Fermentation Three after lectin-affinity chromatography. SDS PAGE. Lane 1-3, sample flow-through; lanes 4-6, wash fractions; lanes 7-8, eluted fractions with 1 M methyl α -D-glucopyranoside. 20 μ l supernatant all wells. Box shows where band would be expected of rmFH (reduced). Arrow: clipped protein. A, protein MWt markers.

Although some rmFH remained attached to the resin, and only eluted with 1 M methyl α -D-glucopyranoside, this appears to represent a smaller proportion of the sample than it did in Fermentation One.

Fractions 1-5 were pooled (~ 134 mg of protein) and exchanged into the equilibration buffer for loading on to an anion-exchange (Source Q) column. Due to the large volume of sample (150 ml), it was divided in two and each sample dialysed separately in 4 L of Source-Q equilibration buffer, twice.

Three Source-Q runs were performed due to the volume of sample that had to be loaded. Figure 3.37 shows a representative chromatogram with SDS PAGE.

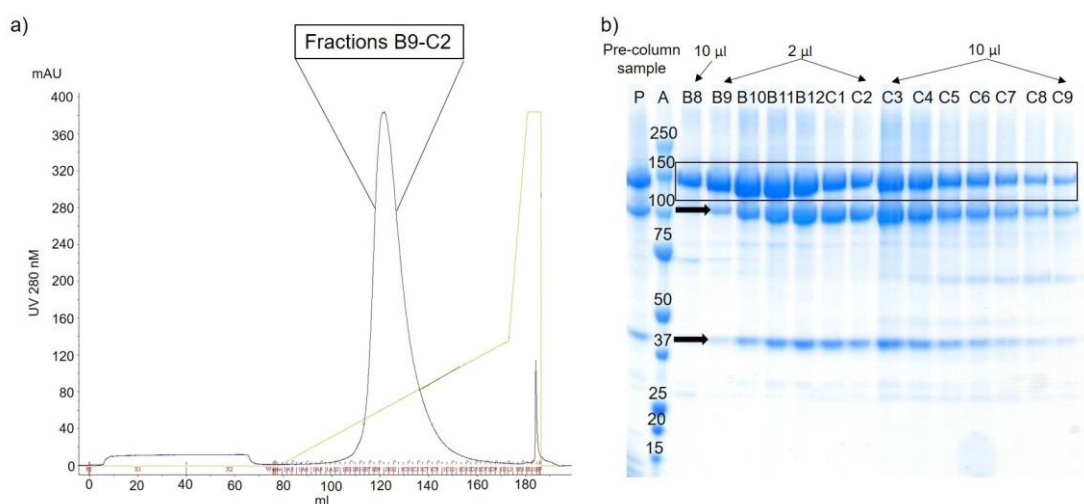


Figure 3.37 RmFH PDI-KM71 Fermentation Three after chromatography on Source Q.
a) Chromatogram from a 0.09 - 1 M NaCl gradient applied over 12 column volumes. b) SDS PAGE (reducing conditions). The box shows where a band would be expected for rmFH. Arrow: clipped protein. P: pre-column sample 10 μ l. B8, C3-C9, 10 μ l supernatant; B9-C2, 2 μ l of supernatant. A, protein MWt markers.

Fractions B9-C2 were pooled from each Source Q run. The total yield of rmFH from Fermentation Three was 78 mg (36 ml at 2.17 mg/ml). This was a 250-fold increase in rmFH production compared to the previous most successful fermentation before PDI (3.1.2.2.3).

3.1.5.3.4 Fermentation Four (pump-fed, anhydrous PMSF)

Despite the encouragingly high yields of rmFH obtained above, clipping remained an issue and the use of PMSF was re-visited. As mentioned above, PMSF rapidly degrades in aqueous solution and the stock must be prepared in anhydrous isopropanol. In previous fermentations, PMSF powder was added directly to the methanol/glycerol feed and might have been hydrolysed even before addition to the fermenter. Fermentation Four was a repeat of Fermentation Two, except that anhydrous methanol and glycerol were used to dissolve anhydrous PMSF.

At harvesting time a reduced biomass of cells compared to Fermentation Three was noticeable, and the fermentation culture was cloudy and green. Figure 3.38 shows the SDS PAGE of supernatant from daily samples during the fermentation process.

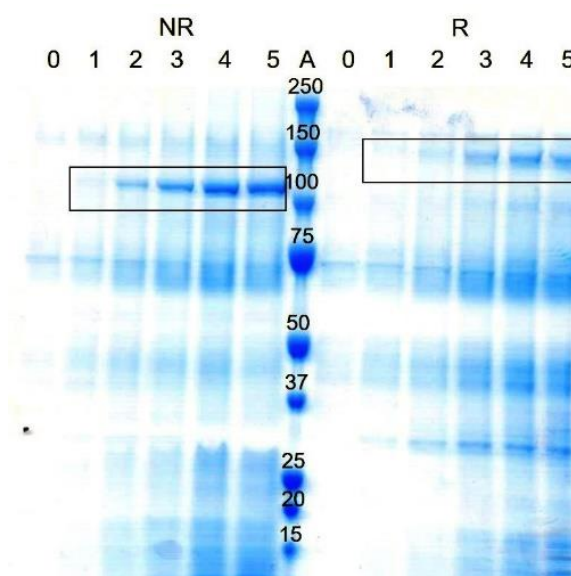


Figure 3.38 SDS PAGE of daily samples Fermentation Four (pump-fed, anhydrous PMSF added). Lanes 0-5, days of fermentation. 0, glycerol feed; 1, methanol induction; 2-4, methanol/glycerol feed days; 5: harvest. NR = non-reducing conditions R= reducing conditions. Box: rmFH. 10 μ l supernatant all wells. A: protein MWt markers.

Figure 3.38 suggests a similar level of rmFH production as was obtained in Fermentation Two (Figure 3.30), which also utilised PMSF. Figure 3.39 shows the chromatogram and SDS-PAGE following the cation-exchange step using Toyopearl SP-650 resin. The absorbance at 280 nm was estimated to be ~280 mAU (peak 340 mAU, baseline 60 mAU), compared to ~310 mAU for Fermentation Two and ~380 mAU for Fermentation Three.

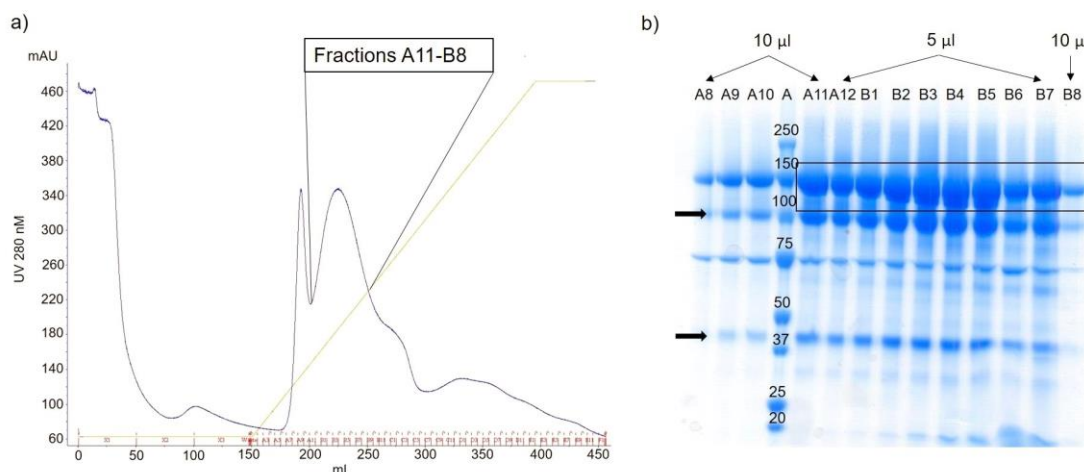


Figure 3.39 RmFH PDI-KM71 Fermentation Four after chromatography on Toyopearl SP-650. a) Chromatogram from a 0.05 - 0.5 M NaCl gradient applied over four column volumes. b) SDS PAGE. Box shows where band would be expected of rmFH (reduced). Arrow: clipped protein. A11, B8, 10 μ l supernatant; A8-A10, A12-B7, 5 μ l of supernatant. The band at ~70 kDa is Endo H α . A: protein MWt markers.

No improvements in clipping was apparent. The estimated protein concentration of the central peak fraction was 2.5 mg/ml (5 ml fraction). As before, fractions with an estimated concentration of greater than 2 mg/ml required dilution by 1:2 prior to further purification. Fractions A11-B8 were pooled to give a total volume of 90 ml (~ 95 mg of protein), then the material was deglycosylated and applied to the lectin-affinity column as before (Figure 3.40).

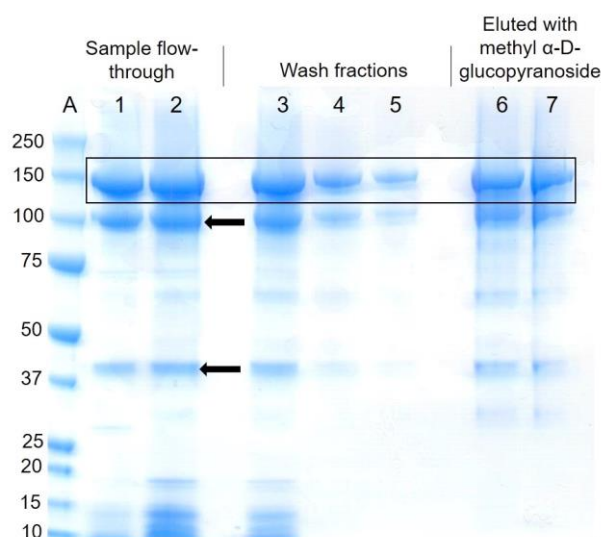


Figure 3.40 RmFH PDI-KM71 Fermentation Four after lectin-affinity chromatography. Lane 1-2, sample flow-through; lanes 3-5, wash fractions; lanes 6-7, eluted fractions with 1 M methyl α -D-glucopyranoside. 20 μ l supernatant all wells. Box shows where band would be expected of rmFH (reduced). Arrow: clipped protein. A: protein MWt markers.

Fractions 1-3 suggests there are more lower-molecular weight bands than were noticed in the post-lectin-affinity SDS-PAGE from Fermentation Three (Figure 3.36). Fractions 1-5 were pooled (120 ml) and buffer-exchanged into the equilibration buffer for the anion-exchange Source Q column. Due to the large volume of sample, it was divided in two and each sample dialysed separately in 4 L of Source -Q equilibration buffer, twice. Two Source-Q runs were performed due to sample volume. Figure 3.41 shows a representative chromatogram and associated SDS-PAGE.

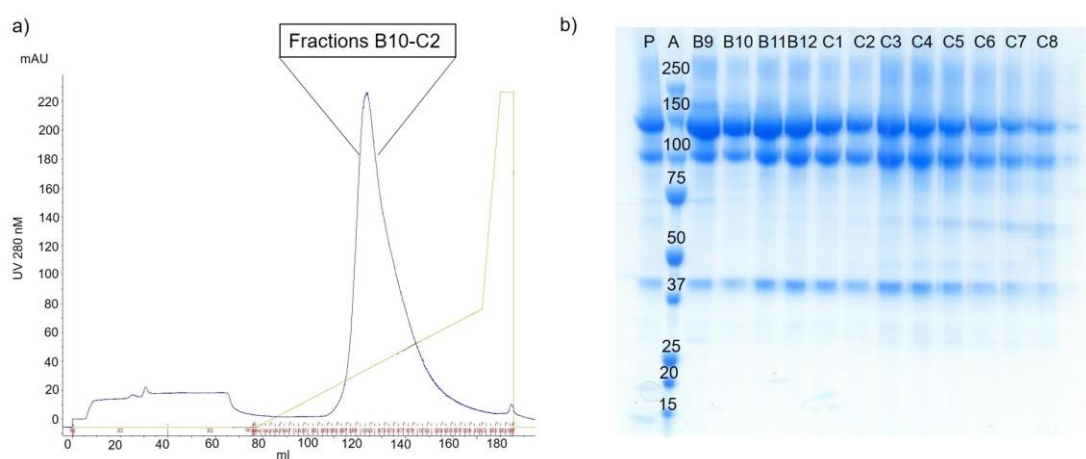


Figure 3.41 RmFH PDI-KM71 Fermentation Four after chromatography on Source Q.

a) Chromatogram from a 0.09 - 1 M NaCl gradient applied over 12 column volumes. b) SDS PAGE. Box shows where band would be expected of rmFH (reduced). Arrow: clipped protein. P, pre-column sample 3 μ l. B9-C8, 3 μ l of supernatant. A: protein MWt markers.

Fractions B10-C2 were pooled from each Source Q run. The total yield of rmFH from Fermentation Four was 29 mg (20 ml at 1.45 mg/ml). All SDS PAGE following Fermentation Four showed more lower-molecular weight bands than Fermentation Three, implying a greater degree of yeast cell lysis in this fermentation.

The Source Q protein samples from all four fermentations were amalgamated (138 mg) and buffer exchanged into phosphate-buffered saline solution (PBS) prior to freezing at -80°C for storage prior to further experiments. The final quantity of purified rmFH was 96 mg (64 ml at 1.5 mg/ml), showing a further loss of protein during the final buffer-exchange step. The combined amount of rmFH protein following the cation-exchange chromatography step for all four fermentations was 378 mg, implying that a final yield of 96 mg is a 75 % loss of protein during the purification process.

3.1.5.3.5 Summary of recombinant murine Factor H PDI-KM71 Fermentations

Table 3.3 summarises the four rmFH PDI-KM71 fermentations with protein yield at each stage.

Fermentation/ Methanol- glycerol feed/ Protease inhibitor	Protein yield following cation- exchange chromatography	Protein yield following lectin affinity and anion-exchange chromatography	Pooled yield following lectin affinity and anion-exchange chromatography	Final yield following buffer exchange into PBS
1 Batch-fed PMSF	21 mg	31 mg	138 mg	96 mg
2 Pump-fed PMSF	96 mg			
3 Pump-fed Pefabloc	166 mg	78 mg		
4 Pump-fed Anhydrous PMSF	95 mg	29 mg		

Table 3.3 Summary of rmFH pJe912 in PDI-KM71 fermentation conditions and protein yield. PBS = Phosphate buffered saline.

3.1.5.4 Purification of recombinant murine Factor H using Ceramic Hydroxyapatite (CHA) Chromatography

As discussed in 2.4.5, CHA chromatography was trialled for rmFH purification in the hope of resolving the remaining lower-molecular weight contaminants. For this trial, Source-Q fractions were used that had not been pooled for the final sample (due to being less pure than fractions from the centre of the peak).

The binding buffer used here was 10 mM sodium phosphate, pH 6.5, 200 mM NaCl. Protein was eluted with either 10 mM sodium phosphate, pH 6.5, 2 M

NaCl, or 500 mM sodium phosphate, pH 6.5, 200 mM NaCl. The reference paper (159) used 5 mM sodium phosphate for plasma FH, but the rmFH precipitated at this concentration and required 10 mM sodium phosphate for stability. Storage of the elution solutions and the chromatography step had to be performed at room temperature to avoid precipitation of the protein solutions in 500 mM phosphate or 2 M NaCl during prolonged cold storage.

Elution with a gradient from 10 mM - 500 mM sodium phosphate yielded a relatively flat chromatographic profile with a hump commencing at ~ 100 mM sodium phosphate and completed by ~ 200 mM sodium phosphate. When eluted fractions were run reduced on SDS PAGE there was no reduction in the lower molecular weight (i.e. clipped) bands compared to a pre-column sample (data not shown).

Figure 3.42 shows the chromatogram and SDS PAGE for an elution with a gradient of 200 mM - 2 M NaCl.

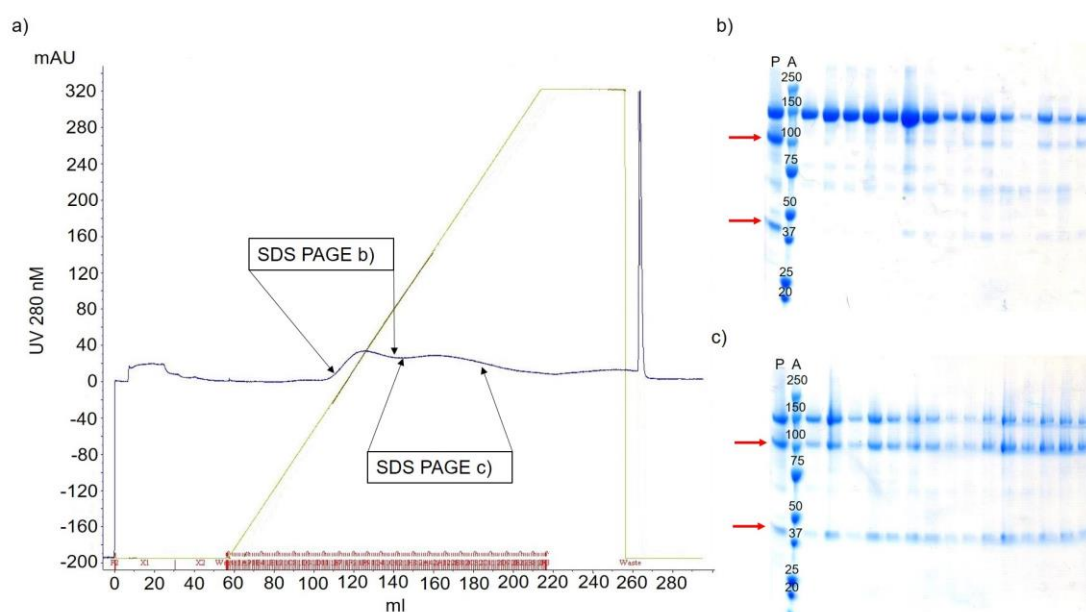


Figure 3.42 RmFH PDI-KM71 after chromatography on ceramic hydroxyapatite

a) Chromatogram from a 200 mM – 2 M NaCl gradient applied over 20 column volumes. b) SDS-PAGE from first protein peak b). c) SDS-PAGE from second protein peak c). Arrows show clipped protein. P: pre-column sample. Alternate fractions run reduced. Fractions were 500 µl each, concentrated using a Vivaspin 500 30 kDa cut-off to ~ 50 µl, with 10 µl loaded per well. A: protein MWt markers.

Figure 3.42 b) shows that the rmFH eluted in the first peak contains markedly less of the lower molecular weight 'clipped' protein bands seen in all previous reduced SDS PAGE. As this SDS-PAGE used alternate fractions (due to the large number of fractions eluted), it was repeated (Figure 3.43) using each fraction.

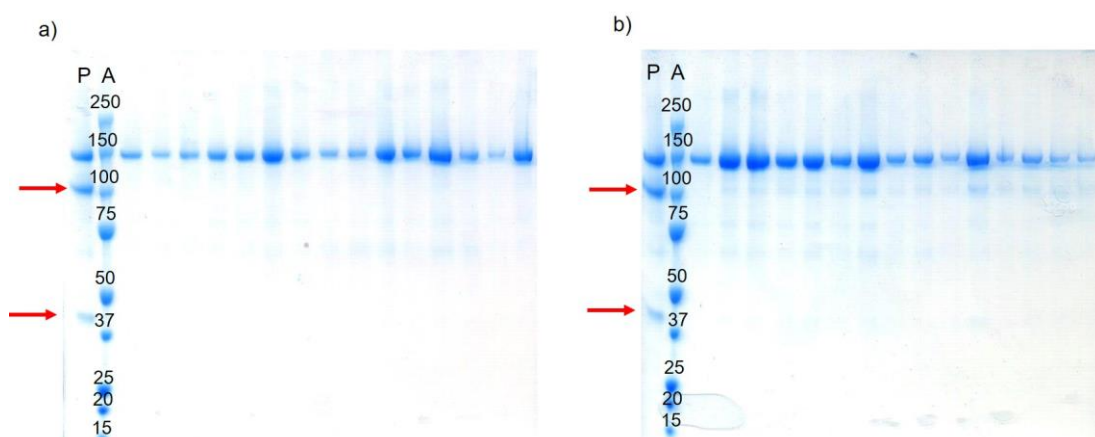


Figure 3.43 RmFH PDI-KM71 after chromatography on CHA. Arrows show clipped protein (or lack of). P: pre-column sample 10 μ l. Fractions were 500 μ l, concentrated using a Vivaspin 500 30 kDa cut-off to $\sim$ 50 μ l, with 10 μ l loaded per well. Run reduced. A: protein MWt markers.

Figure 3.43 a) in particular shows a major reduction in 'clipped' lower-molecular weight bands. It was hoped that using CHA as a final purification step would provide sufficient amounts of non-clipped rmFH for planned *in vivo* experiments. The purification was repeated several times (varying from $\sim$ 6 – 17 mg of protein applied to column) but proved inconsistent. The yield of this chromatographic step was poor corresponding to $\sim$ 17 % of the protein applied to the column (e.g. 16.5 mg of protein applied to the column yielded just 2.9 mg of intact protein; 6.6 mg applied to column gave 1.12 mg). The eluted fractions had a low protein concentration ($<$ 0.05 mg/ml) and further protein was lost during the concentration process/buffer exchange into PBS. It was judged therefore that purification of intact protein in the quantities required for mouse studies was not practical. The final yield of non-clipped rmFH was 2.41 mg (2.41 mg/ml $\times$ 1 ml) and is denoted hereafter as rmFH-CHA.

3.2 Murine Factor H from serum

Purchased mouse serum (100 ml) was split into four batches of 25 ml for mFH purification. Following incubation with PEG, centrifugation to form a pellet, dialysis into an appropriate buffer and filtration using a sterile filter, the protein solution was applied to two 5-ml HiTrap Heparin HP columns connected in tandem. Figure 3.44 shows a representative chromatogram and SDS-PAGE. Due to prolonged washing of the column prior to elution, and for clarity, only a partial chromatogram is shown.

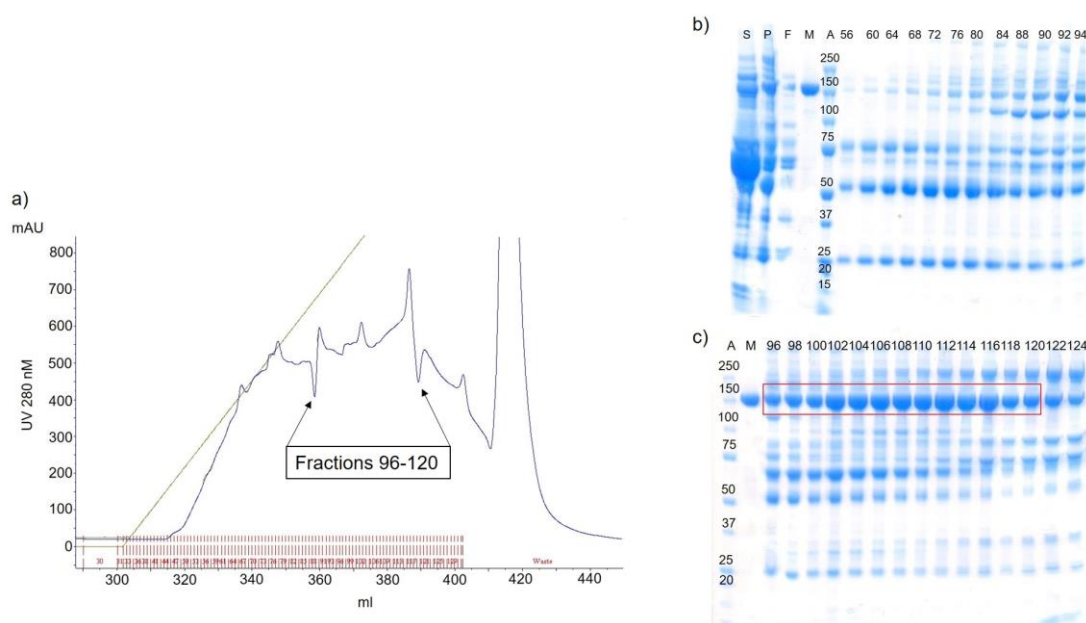


Figure 3.44 Preparation of mFH, HiTrap heparin-affinity chromatography. a) Chromatogram from a 0-250 mM NaCl gradient applied over ten column volumes. b) and c) SDS-PAGE gels: S, mouse serum (1 μ l); P, pre-column sample (2 μ l); F, flow-through (10 μ l); M, purified mFH (10 μ l) (gift from Dr Kevin Marchbank). An aliquot of 10 μ l was loaded for each fraction (numbered above each lane) and run under reducing conditions. Box = fractions pooled. A: protein MWt markers.

A Western blot confirmed the fractions containing mFH (data not shown). The pooled fractions were dialysed and applied to a Source-Q chromatography column. Figure 3.45 shows the results of a representative chromatogram and SDS PAGE.

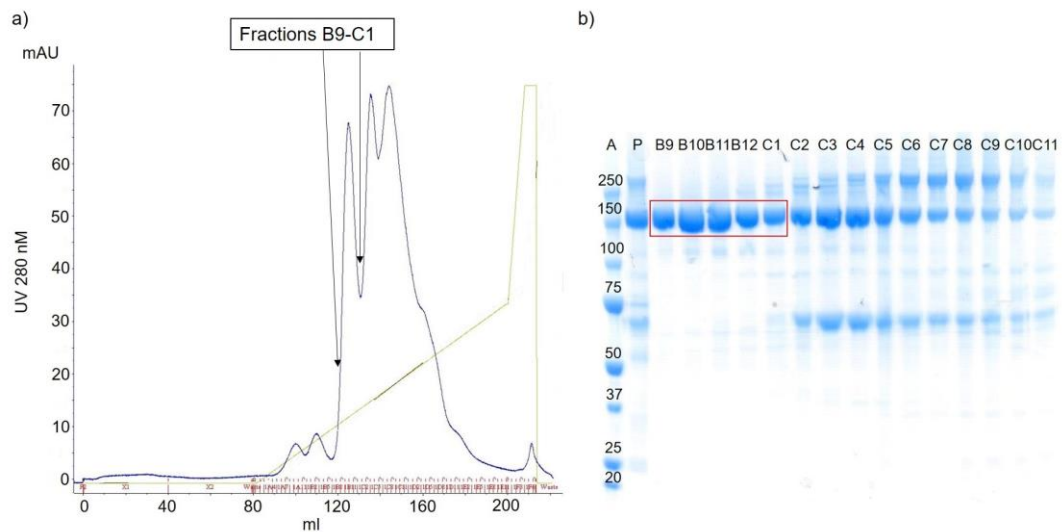


Figure 3.45 MFH, after Source-Q chromatography. a) Chromatogram from a 90 mM -1 M NaCl gradient applied over twelve column volumes. b) SDS PAGE. P, pre-column sample (10 μ l). Fractions (10 μ l each) run under reducing conditions. Box = fractions pooled. A: protein MWt markers.

A Western blot confirmed the fractions containing mFH (data not shown). 100 ml of murine serum yielded 4.9 mg of pure mFH (1.82 mg/ml x 2.7 ml). Ding *et al* (83) found a value of ~ 200 μ g/ml mFH in mice of C57 lineage, although firm data on the concentration of mFH in the serum of mice (from a C56BL/6 background) is not available. This implies a yield of ~ 25 %. These low numbers emphasise the potential usefulness of recombinant rmFH.

Figure 3.46 shows an SDS-PAGE of the final purified samples of rmFH, rmFH-CHA (*i.e.* after purification on hydroxyapatite) and mFH from serum, used in the subsequent characterisation and in vivo experiments.

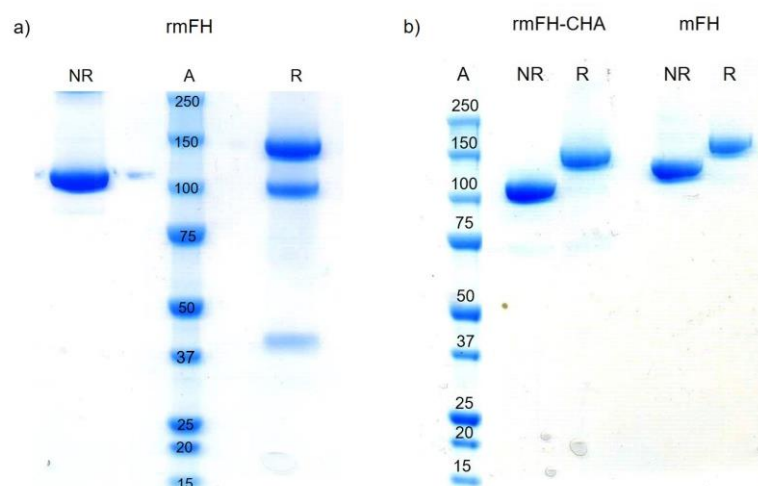


Figure 3.46 SDS PAGE of final samples of purified murine FH. a) 1.5 μ l of 1.5 mg/ml protein solution per well (total 20 μ l with PBS addition). b) 1 μ l of 1.5 mg/ml protein solution per well (total 20 μ l with PBS addition). NR, non-reducing conditions; R, reducing conditions; PBS, phosphate buffered saline. A: protein MWt markers.

3.3 Recombinant human Factor H

3.3.1 Recombinant human Factor H – VYE and IYD polymorphisms

The common haplotypic variants of human FH, VYE (V62-rhFH) and IYD (I62-rhFH), were produced as previously described (23). The final quantity of V62-rhFH produced was 28 mg (1.42 mg/ml x 20 ml) and the purity can be judged from Figure 3.47.

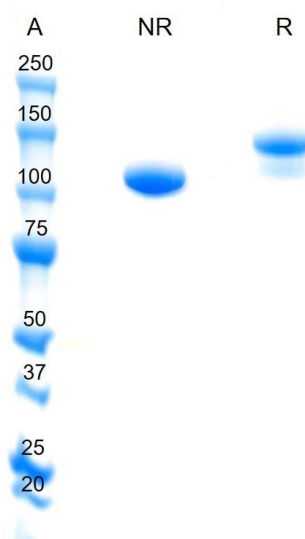


Figure 3.47 SDS PAGE final sample purified V62-rhFH. 20 μ l of 0.2 mg/ml protein solution per well. NR, non-reducing conditions; R, reducing conditions. A, protein MWt markers.

I62-rhFH was produced by Dr Andrew Herbert and its behaviours on SDS-PAGE can be seen in Figure 3.51.

3.3.2 Recombinant human Factor H – D1119G mutation

Growth of cultures, a ten-litre fermentation, and harvesting, were all undertaken as previously described (Section 2.2.7.1). Figure 3.48 shows the chromatogram and SDS-PAGE from the SP-Sepharose cation-exchange step.

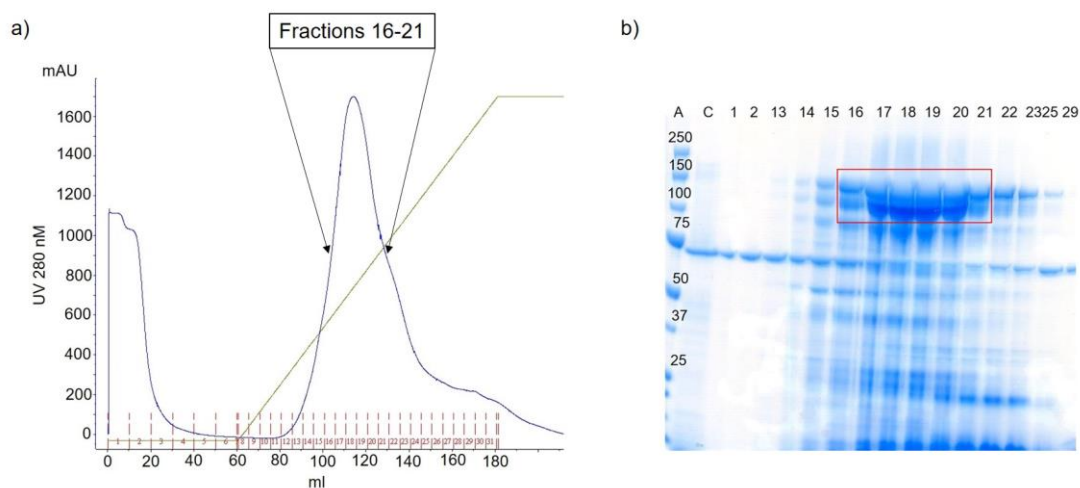


Figure 3.48 Recombinant human factor H mutant D1119G, following chromatography on SP-Sephrose. a) Chromatogram following 0.05 - 1 M NaCl gradient over five column volumes. b) SDS-PAGE. Box shows bands expected to be rhFH (reduced). C, crude supernatant; lanes 1-2, column flow-through. The band at ~70 kDa is Endo H_f. Aliquots of 10 μ l of supernatant were loaded in all wells. A: protein MWt markers.

Fractions 16-21 were pooled and subjected to deglycosylation as previously described, followed by lectin-affinity chromatography (data not shown). The protein solution was buffer-exchanged and applied to a Source-Q anion-exchange column as previously described. Figure 3.49 shows a representative chromatogram and SDS-PAGE. This is for one of four identical runs.

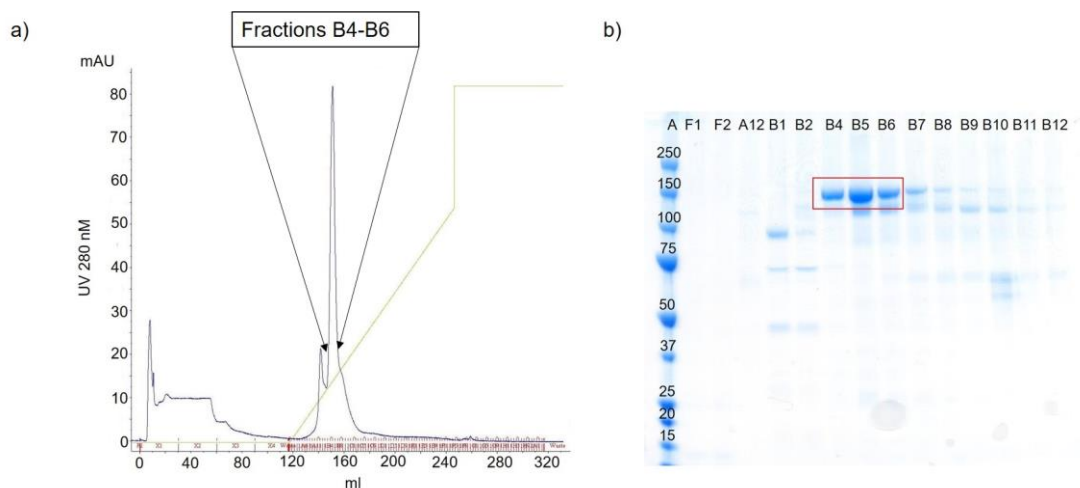


Figure 3.49 Recombinant human factor H mutant D1119G after Source-Q chromatography. a) Chromatogram obtained for a 0.09-1 M NaCl gradient over 12 column volumes. b) SDS PAGE. F1/2, flow-through from column; other lanes, fractions as numbered (10 μ l loaded for each fraction), run under reducing conditions. Box = fractions pooled.

Fractions B4-6 were pooled in each of the four runs, giving 20 ml at 0.22 mg/ml (~ 4.5 mg of protein). Following concentration, only 1.2 mg remained (1.5 ml x 0.8 mg/ml). Figure 3.51 shows the final protein sample. The final yield appears poor given that the SDS PAGE in Figure 3.48b) showed a promising yield of rhFH following the first purification step. As seen with rmFH, a significant proportion of the protein was only eluted from the lectin-affinity column following elution with 1 M methyl α -D-glucopyranoside. In addition, inadvertent use of a lower NaCl concentration solution during buffer exchange for Source Q resulted in precipitation of part of the protein sample. In hindsight a lower number of Source Q runs could have been performed, as this would result in a less dilute final protein solution and avoid the need for losses linked to concentration prior to use in assays.

3.3.3 Recombinant human Factor H – S1191L V1197A and R53H mutations

Growth of starter cultures, fermentation and harvesting were performed as previously described (2.2.7.3). These proteins were produced using a four-day methanol/glycerol continuous-pump feed with the addition of Pefabloc (as in Section 3.1.5.3.3). A total of 3 L of supernatant, harvested from the fermenter, served as the starting material for both purifications described below.

These proteins were harvested using an affinity-purification step (developed by Dr Andrew Herbert) that takes advantage of the very high and selective affinity for human FH of the bacterial binding protein PspCN (160). A PspCN-affinity resin was made (Section 2.4.6) and mixed with 1 L of the harvested supernatant, before loading on a bench-top column and eluting with 100 mM glycine, pH 2.5. Figure 3.50 shows a representative SDS-PAGE of eluted fractions from LA-rhFH (*i.e.* the mutant with Leu at 1191 and A at 1197).

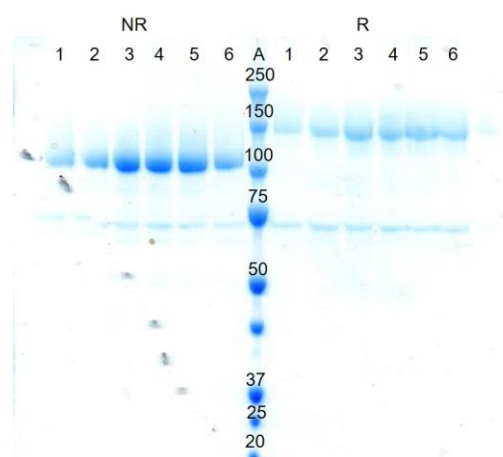


Figure 3.50 SDS-PAGE of human recombinant mutant LA-FH after PspCN-affinity column. 10 μ l of protein solution was loaded per well. Lanes 1-6, eluted fractions. NR, non-reducing conditions; R, reducing conditions. The band at ~70 kDa is Endo H_f. A: protein MWt markers.

In Figure 3.50, the lower-MWt contaminants evident in Figure 3.48b (after cation-exchange chromatography) are absent. This affinity-based method for FH purification is thus advantageous in terms of the purification achieved, and it is doubly so due to the significantly reduced time for completion ($\sim \frac{1}{4}$ of the time needed for cation-exchange chromatography).

Fractions containing rhFH as judged from SDS-PAGE were pooled, buffer exchanged and deglycosylated before undergoing lectin-affinity purification (not shown). As in previous fermentations, a proportion of the protein was only eluted from the lectin-affinity column following elution with 1 M methyl α -D-glucopyranoside. Protein-containing fractions were pooled, buffer-exchanged and subjected to anion-exchange chromatography as a final purification step (not shown).

The final amount of protein (obtained from 3 L of harvested fermentation supernatant) for LA-rhFH was 10.5 mg (1.75 mg/ml $\times$ 6 ml) and for R53H-rhFH it was 4.4 mg (0.73mg/ml $\times$ 6 ml). Figure 3.51 shows the purity of the LA-rhFH, R53H-rhFH, D1119G-rhFH plus I62-rhFH proteins.

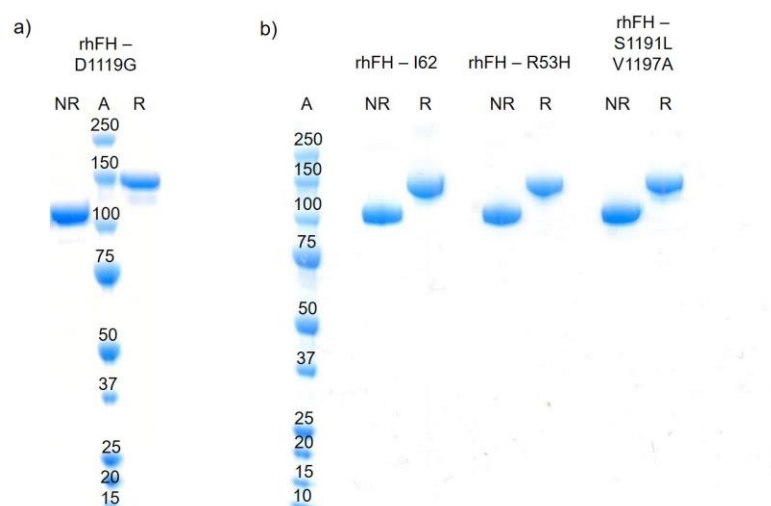


Figure 3.51 SDS PAGE of purified samples of recombinant human FH – I62, R53H, S1191L V1197A. An aliquot of 4 μ l of a 1 mg/ml protein solution, made up to 15 μ l with PBS, was loaded in each well. NR, non-reducing conditions; R, reducing conditions. A: protein MWt markers.

3.3.4 Summary of recombinant human Factor H production

Table 3.4 summarises the production of rhFH with fermentation variables and yields.

rhFH variant	Length of induction (days)	Induction Feed	Method of Induction Feed	Protease inhibitor	Final yield of rhFH
V62	4	Methanol	Batch-fed	PMSF	28 mg
D1119G	3	Methanol	Batch-fed	PMSF	4.5 mg (1.2 mg following concentration)
S1191L V1197A	4	Methanol/glycerol	Pump-fed	Pefabloc	10.5 mg (from 3 L supernatant)
R53H	4	Methanol/glycerol	Pump-fed	Pefabloc	4.4 mg (from 3 L supernatant)

Table 3.4 Summary of rhFH fermentation conditions and protein yield.

3.4 Discussion

Since its introduction as an expression host over 25 years ago (153-155), *P. pastoris* has been exploited to make hundreds of recombinant proteins. It is an expression host capable of growing in simple media, to a high cell density and rapidly producing gram-quantities of recombinant protein. It is advantageous in that it avoids contamination with oncogenic or viral nucleic acids as can occur with proteins prepared from mammalian cells, and there are no toxic cell-wall pyrogens as can be generated by *E. coli*.

In the previously published production of recombinant human Factor H (23), a codon-optimised gene had been placed under the control of the strong, methanol-inducible *AOX1* promoter. To ensure the rhFH enters the secretory pathway, the utilised vector pPICZ α B exploits the *S. cerevisiae* α -mating factor pre-propeptide signal leader sequence that is cleaved during export by the sequential actions of signal peptidases (in the endoplasmic reticulum) and Kex2 endopeptidase and Ste13 dipeptidyl aminopeptidase (in the Golgi). Secretion into the culture media from the transformed KM71H cells made harvesting of rhFH and purification relatively straightforward.

The prior work on recombinant human Factor H in *P. pastoris* had been promising but yields of rhFH were orders of magnitude lower than what had been achieved for many other recombinant proteins. Nevertheless, it was hoped that the use of a codon-optimised gene for rmFH (designed and synthesised by GeneArt, who also provided the rhFH gene) in pPICZ α B would yield at least enough rmFH for characterisation studies. However despite successful cloning of the rmFH gene into pPICZ α B, there was no convincing evidence of protein production from transformed KM71H cells.

An alternative codon-optimised gene was purchased (DNA2.0, now ATUM). The new gene was supplied in a *P. pastoris*-compatible vector pJe912, which like pPICZ α B utilises Zeocin resistance for positive selection and contains DNA encoding the alpha-factor secretion-signal. Different organisms exhibit bias towards using certain codons over others for the same amino acid, and

therefore codon-optimization should boost yields of recombinant protein in a heterologous system. The optimisation algorithms used by various commercial organisations are proprietary but a founder of DNA2.0 reported on the key considerations in optimising DNA sequences for protein production in *E. coli* (197, 198). They found that this was strongly dependent on the choice of codons. The most favourable codons were found to be those read by transfer RNAs that were most highly charged when supplies of amino acids are limiting, rather than codons that are most abundant in highly expressed *E. coli* genes. The DNA2.0 gene sequence shared only 80% identity with the GeneArt sequence.

Nonetheless, production of protein encoded by the DNA2.0 rmFH gene in KM71H cells was judged to be very poor because the cell media had to be concentrated, or the rmFH captured on a heparin column, in order to obtain detectable quantities (Section 3.1.2.1.1). A series of five- or ten-litre fermentations were undertaken in an attempt to optimise production levels. Various parameters were varied including the growth conditions and the strain of *P. pastoris* used (Sections 3.1.2.2.1-3.1.2.2.5, 3.1.3 and 3.1.4), but none of these yielded more than a mg of protein at the end of the purification process. It was clear that, even with refinement of the purification process, the 10s of mg, and preferably about 100 mg, of rmFH required for a robust *in vivo* study using mouse models of disease would not be achievable using this strategy.

It was noted throughout the processes described above that when KM71H cells were cultured in higher concentrations of Zeocin, the production of rmFH became lower (e.g. Figure 3.11). Transformed colonies able to grow on agar plates at higher Zeocin concentrations likely owe their greater resistance to the incorporation of more copies of the Zeocin-resistance gene and hence more copies of the gene for rmFH. This higher copy number should result in increased protein production provided no other factor limits production. But this relationship did not seem to apply here and we sought an explanation.

A review of the literature showed that whilst *P. pastoris* has been used in many instances to make disulphide-containing extracellular proteins, none of these

contain as many disulphides (40) and domains (20) as Factor H does. Factor H is an example of a very large category of disulphide-rich multi-domain proteins that are challenging to produce even in mammalian cells, and have largely resisted attempts at recombinant production in microbes. To consider why overproduction of these proteins is so difficult, we need to consider the demands that their synthesis poses to the expression host.

In eukaryotes, nascently translated polypeptide chains destined for secretion are translocated into the ER where the redox environment favours formation of disulfide bonds. Within the ER, protein disulfide isomerase (PDI) speeds up the native folding of proteins. It achieves this primarily by lowering the energy barrier between disulfide-linked cysteine residues and free cysteine residues within a polypeptide (199). A more rapid exchange between oxidised and reduced forms of cysteine increases the rate of isomerization and hence the speed with which the folding process searches out and finds the lowest energy form of a protein. This normally corresponds to the native pattern of disulfide formation. In addition PDI behaves as a chaperone by inhibiting the aggregation of mis-folded proteins (200). Correctly folded and disulfide-bonded proteins in the ER are transferred to the Golgi and ultimately secreted. An insufficiency of PDI activity allows 'mis-folded' conformations with non-native disulphide bonds to persist. Mis-folded proteins trigger the linked quality-control pathways of the unfolded protein response (UPR), which includes cessation of ribosomal translation and ER-associated protein degradation (201).

Each of the 20 individually folding domains of factor H probably needs to fold successfully within the endoplasmic reticulum for the protein to pass the various "quality checks" and get secreted into the medium. Each domain contains four cysteines forming two disulphide bonds. Without assistance from chaperone proteins (202), a proportion of such domains will be hampered from folding efficiently by formation of non-native disulphide-bridges. The more domains a protein has, the more likely it is that at least one of the domains will

have folding problems and this may be sufficient to prevent secretion and trigger the UPR.

This seems to fit with the observation that yields of recombinant FH-derived proteins correspond to the number of modules they contain. A fragment of hFH consisting of just its C-terminal pair of modules (FH19-20) was produced in 100s-of-mg quantities in a 10-L fermentor (24) of KM71H cells whilst hFH 1-4 was produced an order of magnitude less well (203), and full-length rhFH was almost an order of magnitude lower still (23). These observations, as well as a decrease in rmFH production when grown in higher concentrations of Zeocin, support the hypothesis that the production of multiple domain proteins in *P. pastoris* overwhelms the disulfide-forming machinery in the ER when attempting to overproduce 20-domain full-length Factor H. Subsequently the UPR kicks in and protein yields are reduced. This led to the decision to add the PDI gene into the base *P. pastoris* strain in an attempt to overproduce PDI to assist in the folding of rmFH. As the PDI gene was contained in a vector that also utilised the *AOX1* promoter, we reasoned that it would be produced when most needed, *i.e.* during the post-methanol induction phase. The codon-optimised genes for PDI and rmFH were inserted into the yeast genome separately, to allow for the production of a versatile base strain (PDI-KM71) that could be utilized for production of other recombinant protein.

It was immediately apparent on fermentation (Section 3.1.5.3.1) that co-expression with *PDI* improved the production of rmFH by orders of magnitude and of rhFH by tenfold. These gains are larger than those observed in other, reported, cases of *PDI* co-expression, presumably because of the uncommonly high number of disulfide-stabilised domains in FH. A further partial optimization of fermentation conditions, including the use of a pump for combined glycerol/methanol feeding, and the use of the protease inhibitor Pefabloc, resulted in the ability to produce hundreds of mg of purified rmFH. This achievement paves the way for the production of other disulphide-rich multi-domain proteins in *P. pastoris*.

Given the previous success in production of rhFH (23), it was not unexpected that reasonable yields were obtained from the fermentations of rhFH. Although V62-rhFH was produced using the non-optimised fermentation method (batch-fed, use of PMSF as a protease inhibitor), it produced an excess of rhFH compared to the mutant rhFH proteins (Table 3.4). Despite lower initial yields however, the use of a novel affinity-purification method for LA-rhFH and R53H-rhFH allowed rapid purification of the protein with less contaminating yeast proteins. Given the results seen in Figure 3.26 with I62-rhFH, it would be advantageous for future production of rhFH mutants to be undertaken using the PDI-KM71 strain, and to be purified via affinity purification on a PspCN-resin.

4: Biochemical and functional characterisation of recombinant murine Factor H

4.1 Mass spectrometry

4.1.1 In-gel trypsin digest

In-gel trypsin digest was performed to confirm production of rmFH. The peptide fragments were analysed by mass spectroscopy against the amino acid sequence for mFH (Uniprot P06909). Figure 4.1 shows that 67% coverage of the protein was achieved which confirms production of rmFH. Note that four out of seven potential N-glycosylation sites were identified on the peptide sequence coverage, meaning there was a GlcNAc residue present on the relevant peptide. Of the three potential N-glycosylation sites not identified, two reside on peptides not covered by the mass spectroscopy result.

```
1 ED CKGPPPPRENS EILSGSWSEQ LYPEGTQATY
51 KCRPGYRTLG TIVKVCKNGK WVASNPSRIC RKKPCGHPGD TPFGSFRLAV
101 GSQFEFGAKV VYTCDDGYQL LGEIDYRECG ADGWINDIPL CEVVKCLPVT
151 ELENGRIVSG AAETDQEYF GQVVRFECS GFKIEGHKEI HCSENGLWSN
201 EKPRCVELC TPRVENGDG INVKPVYKEN ERYHYKCKHG YVPKERGDAV
251 CTGSGWSSQP FCEEKRCSP YILNGIYTPH RIIHRSDDEI RYECNYGFYP
301 VTGSTVSKCT PTGWIPVPRC TLKPCEFPQF KYGRLYEES LRPNFPVSIG
351 NKYSYKCDNGFSPPSGYSWD YLRCTAQQWE PEVPCVRKCV FHYVENGDSA
401 YWEKVYVQGG SLKVQCYNGY SLQNGQDTMT CTENGWSPPP KCIRIKTCSA
451 SDIHIDNGFL SESSSIYALN RETSYRCKQG YVTNTGEISG SITCLQNGWS
501 PQPSCIKSCD MPVFENSITK NTRTWFKLND KLDYECLVGF ENEYKHTKGS
551 ITCTYYGWSD TPSCYEREC VPTLDRKLVV SPRKEYRVG DLLEFSCHSG
601 HRVGPDSVQC YHFGWSPGFP TCKGQVASCA PPLEILNGEI NGAKKVEYSH
651 GEVVKYDCKP RFLKGPNNI QCVDGNWTTL PVCIEEERTC GDIPELEHGS
701 AKCSVPPYHH GDSVEFICEE NFTMIGHGSV SCISGKWTQL PKCVATDQLE
751 KCRVLKSTGI EAIKPKLTEF THNSTMDYKC RDKQEYERSI CINGKWDPEP
801 NCTSKTSCPP PPQIPNTQVI ETTVKYLDGE KLSVLCQDNY LTQDSEEMVC
851 KDGRWQSLPR CIEKIPCSQP PTIEHGSINL PRSSEERRDS IESSSHEHGT
901 TFSYVCDDGF RIPEENRITC YMGKWSTPPR CVGLPCGPPP SIPLGTVSLE
951 LESYQHGEEV TYHCSTGFGI DGPAFICEG GKWSDPPKCI KTDCDVLPTV
1001 KNAIIRGKSK KSYRTGEQVT FRCQSPYQMN GSDTVTCVNS RWIGQPVCKD
1051 NSCVDPPHVP NATIVTRTKN KYLHGDRVRY ECNKPLELFG QVEVMCENGI
1101 WTEKPKCRDS TGKCGPPPI DNGDITSLSL PVYEPLSSVE YQCQKYLLK
1151 GKKTITCTNG KWSEPPTCLH ACVIPENIME SHNILKWRH TEKIYSHSGE
1201 DIEFGCKYGY YKARDSPFPR TKCINGTINY PTCV
```

Figure 4.1 Results of recombinant murine Factor H in-gel trypsin digest and MS peptide analysis. Text in red denotes sequence coverage. Text in blue denotes identified GlcNAc sites. Text in green denotes non-identified GlcNAc sites (bold for MS covered peptide).

4.1.2 Mass of the intact protein

For rmFH, the calculated molecular weight is 138745 Da. This is based on totalling the molecular weights of: the amino acid residues that make up the primary sequence (Uniprot P06909, 136984 Da); the expected N-terminal artefact arising from incomplete Ste13 cleavage of the signal sequence

(EAEA, 420 Da); and the seven GlcNAc residues remaining after EndoH cleavage of N-glycans (each of 203 Da); minus 80 Da to account for the 40 expected disulphide bonds (due to the loss of two hydrogens). Figure 4.2 shows the result of MALDI-TOF intact mass analysis. This includes one species of 139000 Da, which could correspond to a single-charged species of the expected mass. A second peak at 71,500 could – within the error of the technique – correspond to a double-charged species.

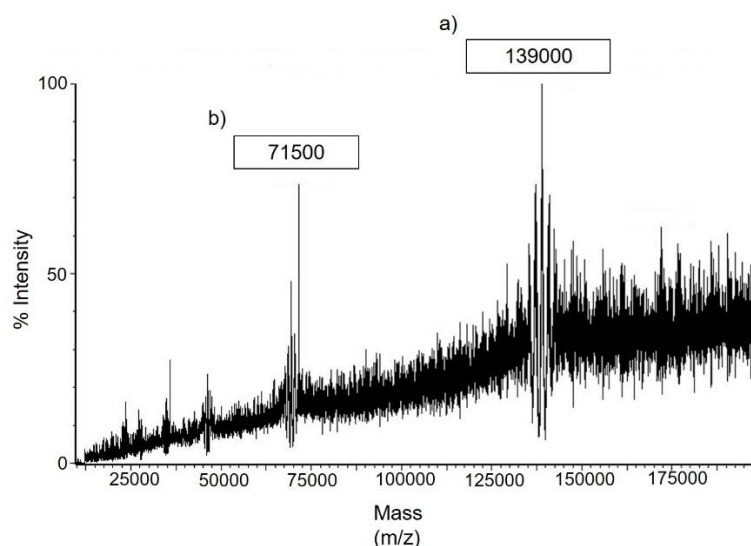


Figure 4.2 Results of recombinant murine Factor H MALDI-TOF spectrometry. a) single-charged species b) double-charged species. m/z: mass to charge ratio.

4.2 Dynamic light scattering

Dynamic light scattering (DLS) was performed to confirm that samples of mFH (both recombinant and purified from serum) were predominantly monodispersed. Oligomers, or other types of aggregate could compromise subsequent *in vitro* and *in vivo* assays. Figure 4.3, 4.4 and 4.5 show the DLS results for rmFH, rmFH-CHA (non-clipped protein) and mFH respectively.

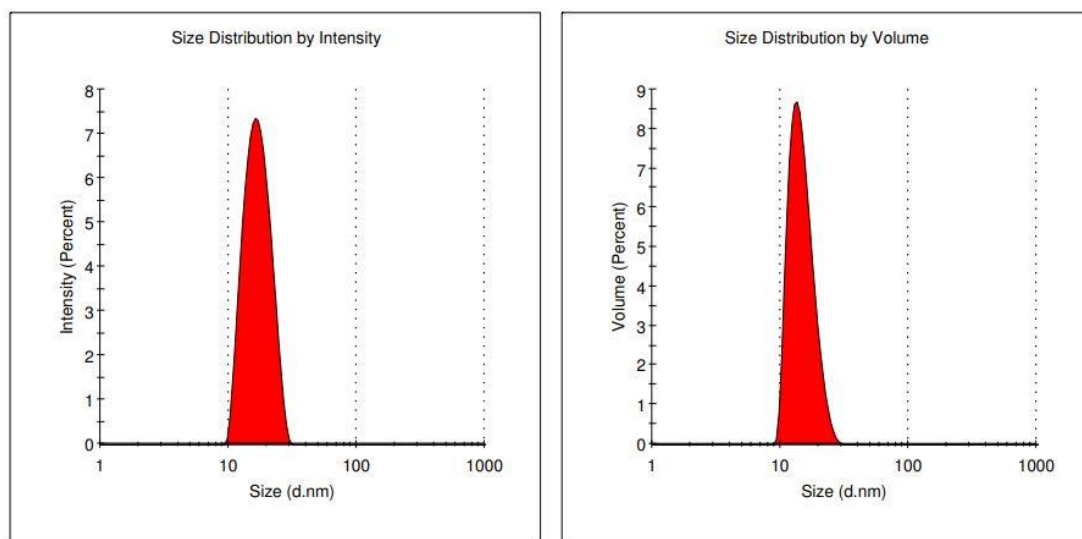


Figure 4.3 DLS data for recombinant murine factor H. Protein concentrations were 1mg/ml in Phosphate Buffered Saline.

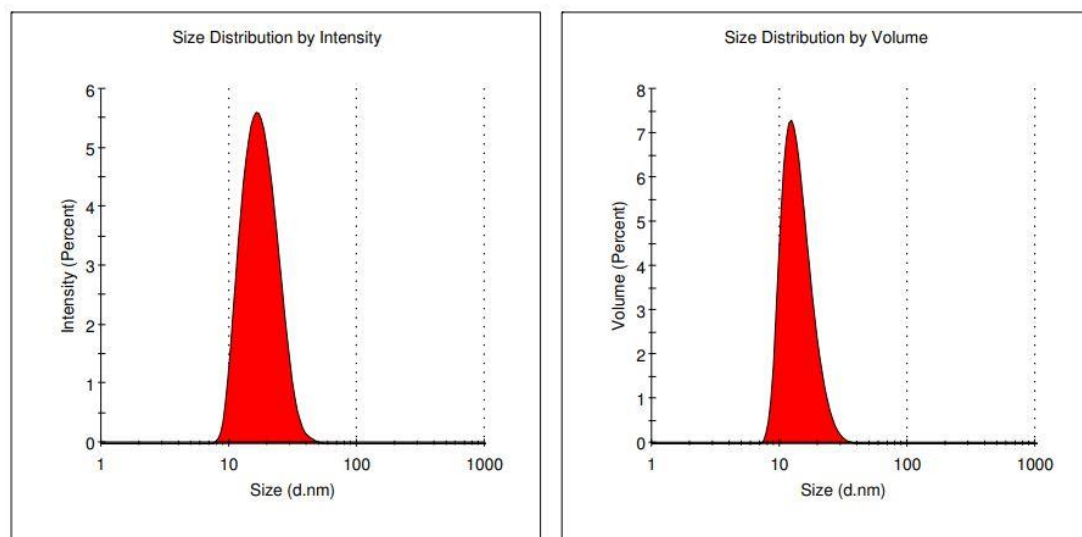


Figure 4.4 DLS data for recombinant murine factor H-CHA. Protein concentrations were 1mg/ml in Phosphate Buffered Saline.

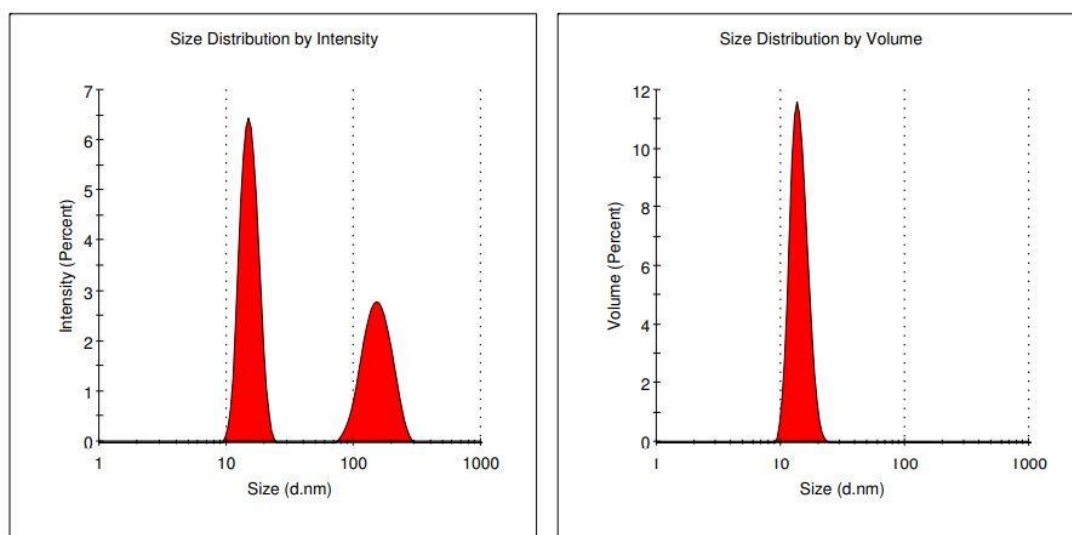


Figure 4.5 DLS data for murine factor H from serum. Protein concentrations were 1mg/ml in Phosphate Buffered Saline.

The values on the x-axis are not easily interpretable in terms of hydrodynamic radius (R_H) but a rule-of-thumb for a highly extended protein is that the $R_H = \text{Mwt (in kDa)} \times 0.6$, suggesting 10-15 nm for FH (204). Of the plots by volume, mFH from serum has the sharpest peak. This is consistent with a lower tendency to self-associate and higher solubility (compared to rmFH), as would be expected due to its native N-glycosylation. Nonetheless, the plot by intensity shows that serum mFH contains a much larger species that is absent in recombinant samples. Its origin and composition are unknown but note that by-intensity DLS plots are extremely sensitive to the presence of even tiny quantities of aggregates (since these give a very much more intense signal than smaller particles). The data for rmFH show an absence of larger particles or multimers.

4.3 N-terminal sequencing

During rmFH purification it became apparent that some of the material was proteolytically damaged or 'clipped' (e.g. Figure 3.46 a). Upon running SDS-PAGE under reducing conditions, in addition to a band for full-length rmFH at ~ 140 kDa, two lower bands appeared at approximately 100 kDa and 40 kDa. N-terminal sequencing was performed on these lower bands to determine at

which position within the rmFH polypeptide cleavage had occurred. The N-terminal sequence of the 100 kDa band was:

E	A	E	A	E	D
Glu	Ala	Glu	Ala	Glu	Asp

This sequence is consistent with the expected inefficient cleaving of EAEA by Ste13 (158) and with Glu and Asp being the N-terminal two amino acid residues in the native mFH sequence.

The N-terminal sequence of the 40 kDa band was:

D	S	I	E	S	S
Asp	Ser	Ile	Glu	Ser	Ser

This sequence lies within the 15th CCP module of mFH. A comparison of the amino acid sequences of hFH CCP15 and mFH CCP15 (by Dr Dinesh Soares of the MRC Human Genetics Unit, University of Edinburgh) showed that the sequence DSI ESS of mFH corresponds to an eight-amino acid residue insertion with respect to hFH and that cleavage has therefore occurred between Arg(872) and Asp(873). Figure 4.6 shows the result of Dr Soares' use of the MODELLER programme (Section 2.6.3) to build a homology model-based three-dimensional structure of mFH CCP 15 (using the hFH CCP15 structure as a template). This shows that the eight-amino acid residue insertion lies within what is probably a disordered region that has been called the "hypervariable loop" (because it is poorly conserved between CCP modules and is often a site of deletions or insertions). Whether this site is also proteolytically sensitive *in vivo* remains unknown but within the context of native glycoprotein it might be shielded by N-glycans (glycans are removed during purification of recombinant material).

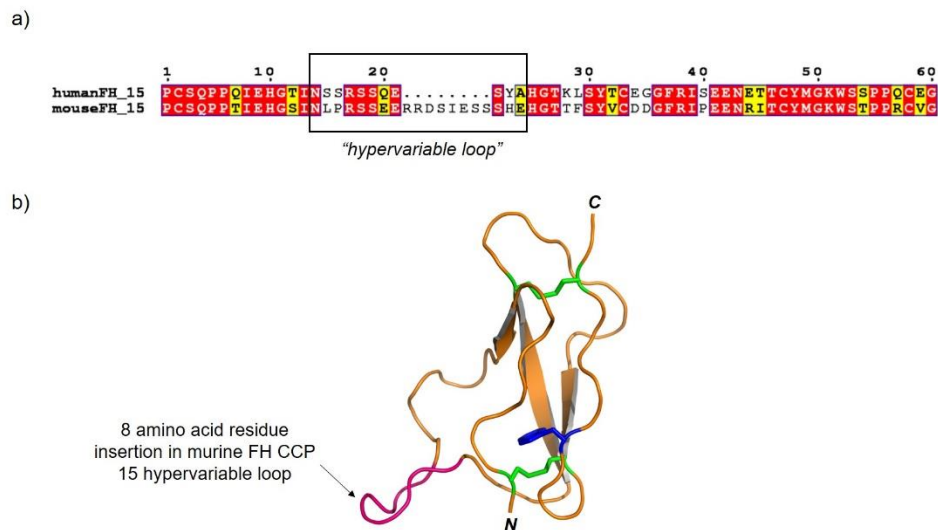


Figure 4.6 Human and murine factor H CCP 15 amino acid sequences. a) Comparison of human and murine factor H CCP 15 amino acid sequence numbered from the first residue of CCP 15. b) Proposed model of murine factor H CCP 15 with hypervariable loop. Green sticks: cysteines forming disulphide bonds. Blue sticks: the invariant (within CCP modules) tryptophan. Figure provided by Dr Dinesh Soares.

4.4 Co-factor assay

Factor H functions as a co-factor for the factor I (FI) mediated proteolytic inactivation of C3b. C3b is comprised of alpha (α) and beta (β) subunits which are joined by disulphide bonds. When C3b is run under reducing conditions on SDS-PAGE these appear as separate bands at 101 kDa and 75 kDa, respectively. Following FI-catalysed cleavage of C3b in the presence of FH, the C3b α subunit splits into fragments of 68 kDa (that, *in vivo*, remains disulfide linked to the β -subunit forming C3c) and 43 kDa (that *in vivo* remains linked by an ester bond to surfaces and is called C3dg) while the β subunit remains intact. C3dg can be further cleaved, releasing C3g (3 kDa) and leaving behind membrane-attached C3d (Figure 4.7 a).

As an initial experiment to investigate the functionality of rmFH, a SDS-PAGE-based co-factor assay was performed using human C3b and human FI (mC3b was not available at this time). Previous work (80) had shown serum-purified mFH to be functional in a co-factor assay using human versions of C3b and FI. As shown in Figure 4.7 b, FI, in the presence of our sample of rmFH,

successfully cleaves C3b α into the two expected iC3b α bands. At the same concentration of FI and hFH/rmFH (300 nM), rmFH appears to function less well than hFH in cleaving C3b α , likely due to the use of human C3b and FI rather than the murine orthologues. This is consistent with previously published work (80).

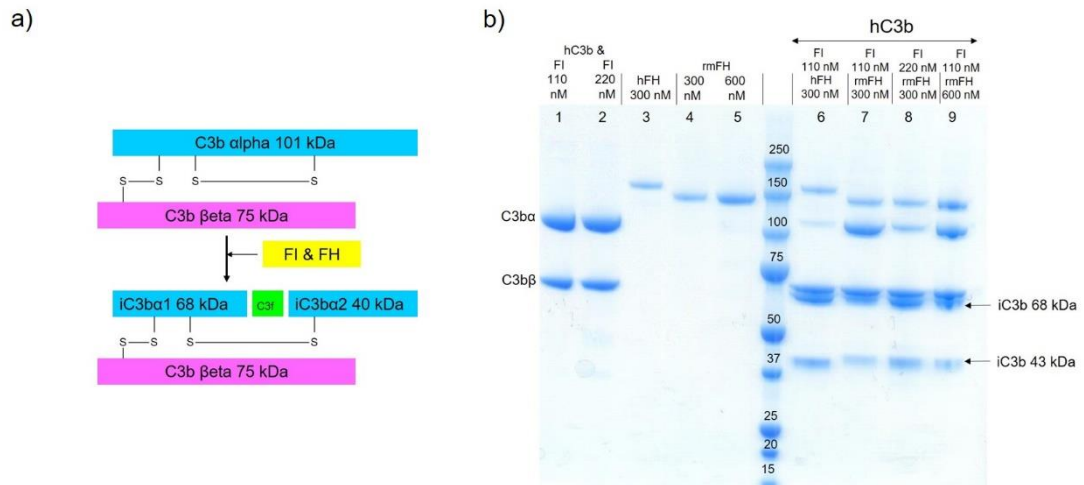


Figure 4.7 Co-factor assay. a) Image illustrating C3b α and β chains with disulphide bonds, and cleavage in the presence of FI and FH. b) 850 nM hC3b used in lanes 1-2, 6-9. Lanes 3-5 contain hFH or rmFH alone at either 300 or 600 nM. Lane 6 contains hFH and FI 110 nM, 7-9 contain rmFH at either 300 or 600 nM, with FI at either 110 nM or 220 nM. RmFH-CHA was used in this assay.

4.5 Surface plasmon resonance

4.5.1 Recombinant mFH binding to human C3b

An initial C3b-binding experiment was performed using human C3b (hC3b, purchased from Complement Technology). Binding to C3b is required both for the factor I co-factor activity of FH and its decay accelerating activity (DAA) towards the C3bBb complex that is the C3 convertase of the alternative pathway. Previous work (80) has shown that mFH modules 1-5 and 18-20 bound to human C3b, although affinity constants were not reported.

Human C3b was amine coupled to a C1 SPR chip (258 response units (RUs), over which was flowed a concentration series (0-2 μ M) of rmFH. Ideally the concentration range should extend to more than twice the expected affinity constant value (K_D) but the series was limited by protein availability. The K_D

was obtained by fitting steady-state binding levels, derived from the background-subtracted traces, to a one-to-one binding model. Figure 4.8 shows the binding of rmFH-CHA (*i.e.* the sample after removal of clipped material) to hC3b, giving a K_D of $1.6 \mu\text{M} \pm 0.19 \mu\text{M}$. This is not far outside of the range of values for binding of hFH to hC3b (K_D s of $0.33 - 1.3 \mu\text{M}$ have been reported (23, 24, 161, 205).

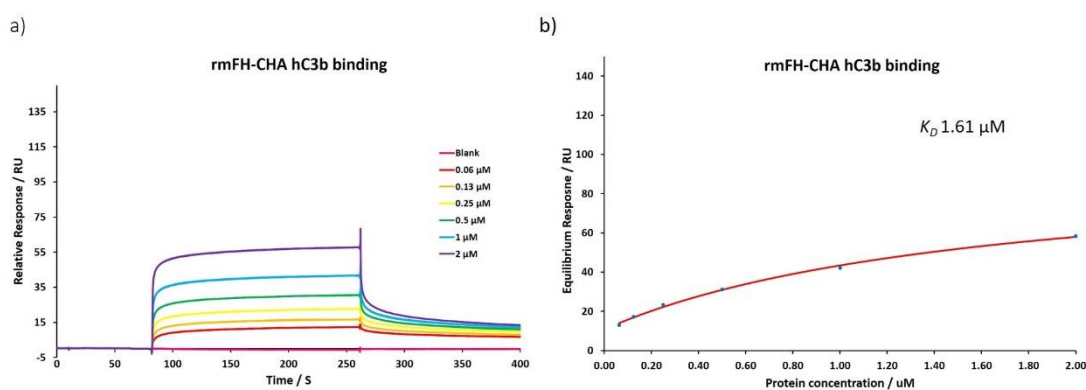


Figure 4.8 Binding of recombinant murine factor H-CHA to human C3b. a) Background-subtracted sensorgram with concentrations as per legend b) Plot of response versus concentration.

Recombinant mFH (*i.e.* the sample before removal of the clipped material) gave a K_D of $2.24 \mu\text{M} \pm 0.49 \mu\text{M}$ (Appendix G). Whilst this may imply that clipped rmFH has a lower affinity for hC3b, further replicates would be required for clarification. Table 4.1 summarises binding data for rmFH and rmFH-CHA with hC3b on a C1 chip.

	K_D (μM)	S.E (μM)	R_{max}	R_{offset}	χ^2
rmFH-CHA	1.61	0.19	85.1	10.7	0.84
rmFH	2.24	0.49	98.4	25.2	2.01

Table 4.1 Summary of binding of rmFH to human C3b. SE: standard error. R : response units. χ^2 : chi².

4.5.2 Murine Factor H binding to murine C3b/d

4.5.2.1 Murine C3b

The binding was measured of rmFH, rmFH-CHA and mFH (purified from serum) to murine (m)C3b on a C1 SPR chip (mC3b was a gift, see Section 2.8.1.1). There appear not to be any affinity data for this interaction reported in the literature. Figures 4.9, 4.10 and 4.11 show the sensorgrams and affinity curves for the binding of rmFH, rmFH-CHA and mFH, respectively, to mC3b (242 RUs immobilized). The binding data are summarised in Table 4.2. The binding of rmFH to mC3b was repeated in a separate experiment and these data are also included in Table 4.2. All affinity constants are estimates as the equilibrium response curves have not reached steady state – a higher top FH concentration would ideally have been used for greater accuracy.

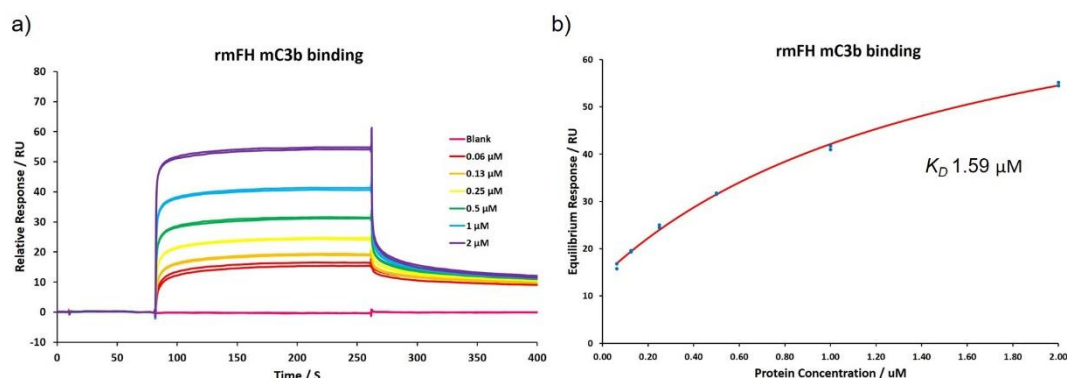


Figure 4.9 Binding of recombinant murine factor H to murine C3b. a) Background-subtracted sensorgrams with concentrations as per legend; b) Plot of response *versus* concentration.

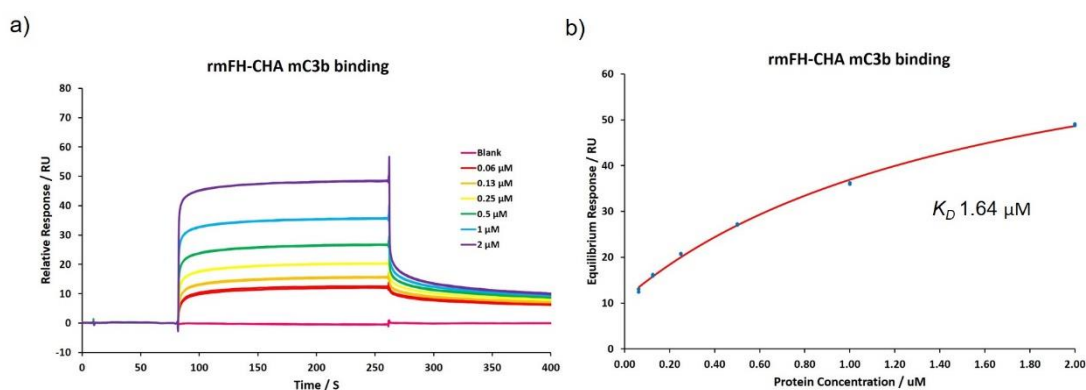


Figure 4.10 Binding of recombinant murine factor H-CHA to murine C3b. a) Background-subtracted sensorgram with concentrations as per legend; b) Plot of response versus concentration

Note that the binding affinity of recombinant murine factor H to murine C3b is similar both in samples containing (rmFH) or not containing (rmFH-CHA) clipped material. The cleavage site is in CCP 15, which does not lie within the C3b-binding regions of mFH.

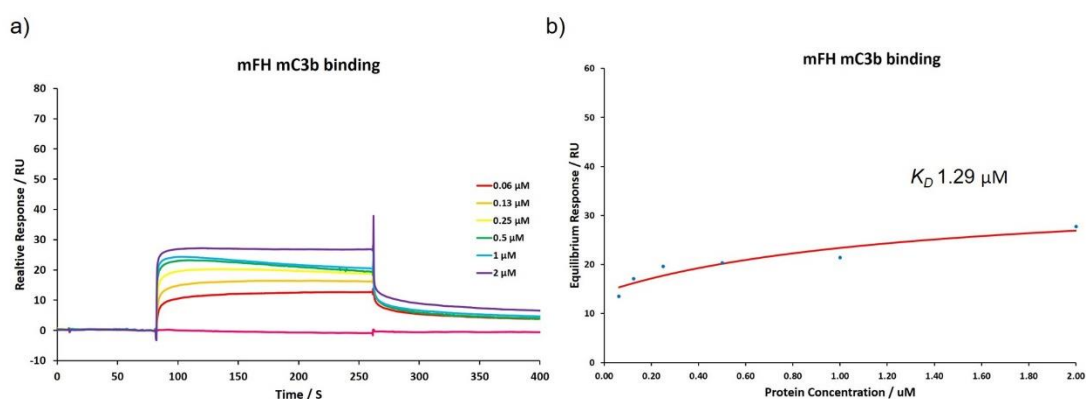


Figure 4.11 Binding of murine factor H to murine C3b. a) Background-subtracted sensorgram with concentrations as per legend b) Plot of response versus concentration

In Figure 4.11 a) there appear to be issues with the traces for 0.5 μM and 1 μM serum-derived murine FH. Moreover, the value of R_{max} is inexplicably lower than for the other versions of mFH. Unfortunately, the very limited stock of mFH prevented a repetition of this experiment. This meant it was not possible to obtain a reliable value of binding affinity between mFH and mC3b although

the value generated from fitting of the experimental data ($1.29 \pm 1.68 \mu\text{M}$) was within the expected range .

	K_D (μM)	$S.E$ (μM)	R_{max}	R_{offset}	χ^2
rmFH	1.59	0.17	72.50	14.08	0.52
	1.18	0.10	86.70	18.66	0.64
rmFH-CHA	1.64	0.17	68.70	10.92	0.47
mFH	1.29	1.68	20.53	14.39	4.03

Table 4.2 Summary of binding of murine factor H to murine C3b. SE: standard error. R : response units. χ^2 : chi².

4.5.2.2 Murine C3d

Previous work showed poor binding of hFH to hC3d immobilized on an SPR chip (160, 161). Similar results might be expected for mFH and mC3d, but there is limited data available in the literature for these binding partners; an exception is the work of Antonioli et al (86), which showed a binding affinity of $3.85 \mu\text{M}$ between mFH and recombinant murine C3d using a CM5 chip and amine coupling of the rmC3d. Figures 4.12, 4.13 and 4.14 show the sensorgrams and affinity curves for the binding of rmFH, rmFH-CHA and mFH respectively to mC3d. The binding data is summarised in Table 4.3. The binding of rmFH to mC3d was repeated in a separate experiment and this data is included in Table 4.3.

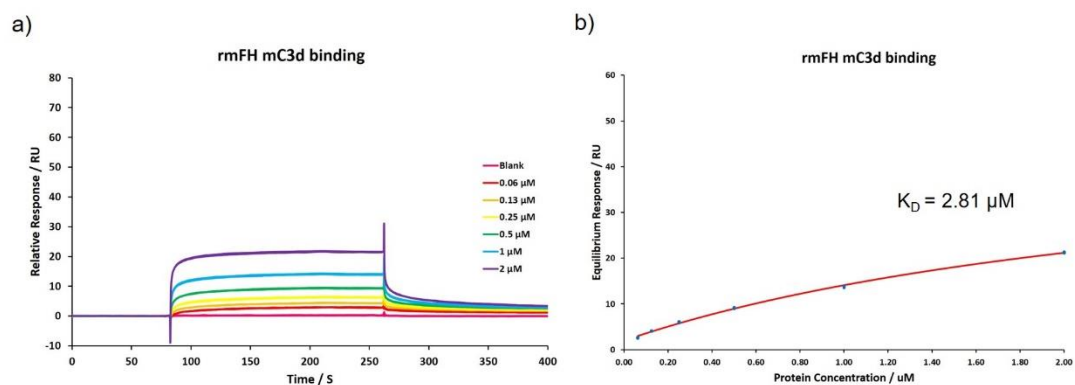


Figure 4.12 Binding of recombinant murine factor H to murine C3d. a) Background-subtracted sensorgram with concentrations as per legend; b) Plot of response versus concentration

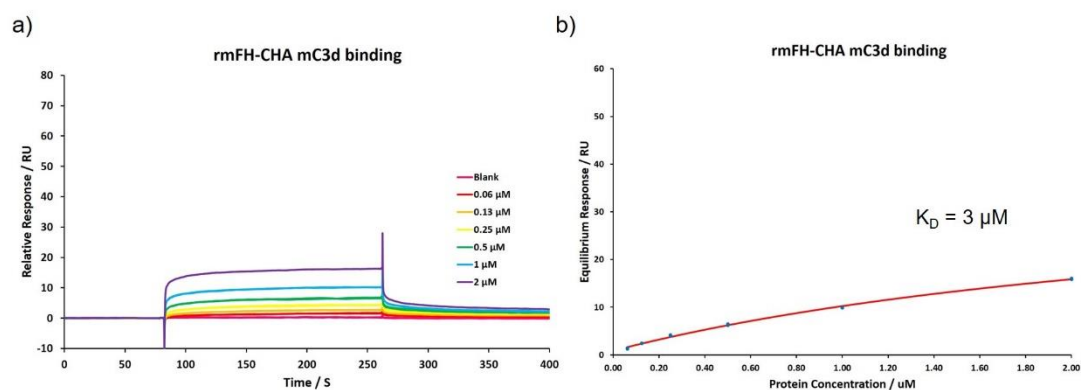


Figure 4.13 Binding of recombinant murine factor H-CHA to murine C3d. a) Background-subtracted sensorgram with concentrations as per legend b) Plot of response versus concentration

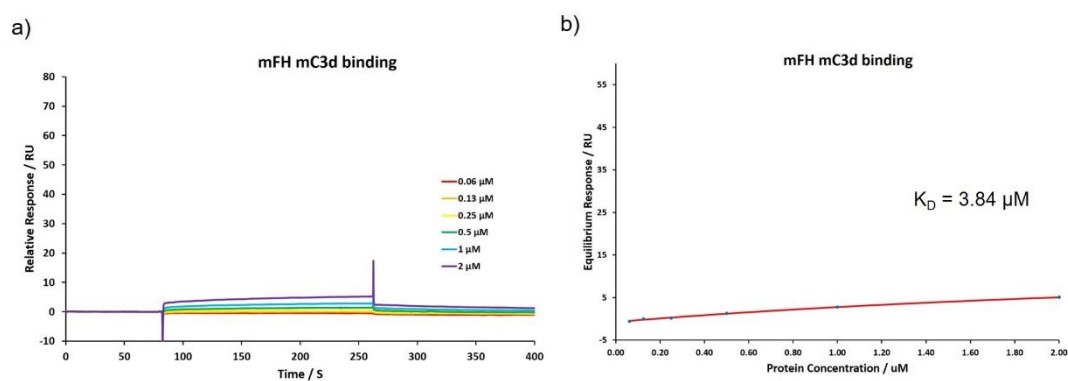


Figure 4.14 Binding of murine factor H to murine C3d. a) Background-subtracted sensorgram with concentrations as per legend; b) Plot of response versus concentration

	K_D (μM)	S.E (μM)	R_{max}	R_{offset}	χ^2
rmFH	2.81	0.37	46.10	1.96	0.10
	2.71	0.32	32.45	2.22	0.04
rmFH-CHA	3.00	0.48	37.50	0.89	0.08
mFH	3.84	1.09	17.00	-0.73	0.02

Table 4.3 Summary of binding of murine factor H to murine C3d. SE: standard error. R : response units. χ^2 : chi².

In all three cases the R_{max} values are lower than we would expect given the initial loading onto the chip of 68 RUs of C3d. As before, all affinity constants are estimates as the highest concentration in the series is below the predicted K_D . Nonetheless, as Table 4.3 shows, the calculated K_D for the binding of mFH to mC3b is similar to that quoted by Antonioli.

4.5.3 Human Factor H binding to murine C3b

Murine C3b has 75% amino acid identity to human C3b. Previous work (83, 138, 139) suggested that human FH is functional in murine *in vivo* studies. Thus it was reasoned that the binding affinity of hFH for mC3b should be similar to that for rmFH and hC3b, or mFH and mC3b. Figure 4.15 shows a representative sensorgram and affinity curve for the binding of hFH to mC3b. This experiment was repeated once, with the data being summarised in Table 4.4. Binding affinities were shown to be comparable to that estimated for rmFH/hC3b and mFH/mC3b.

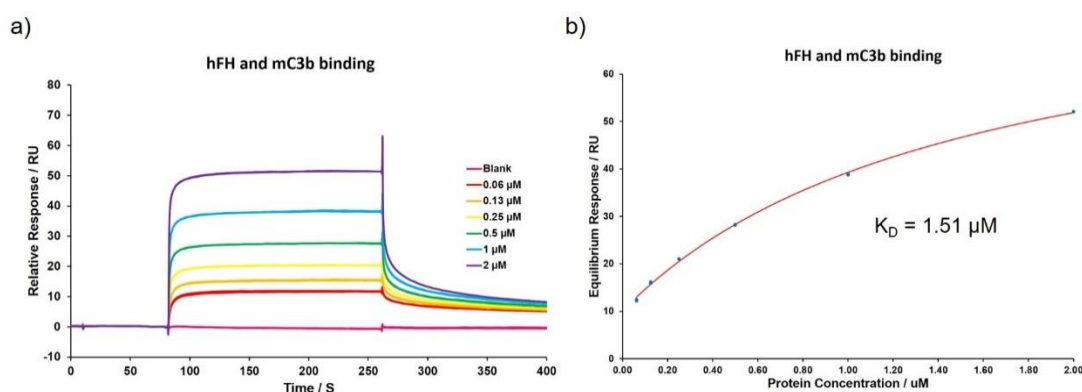


Figure 4.15 Binding of human factor H to murine C3b. a) Background-subtracted sensorgram with concentrations as per legend, b) Plot of response versus concentration

	K_D (μM)	S.E (μM)	R_{max}	R_{offset}	χ^2
hFH	1.51	0.11	73.5	10.1	0.25
	1.34	0.15	61.1	13.9	0.29

Table 4.4 Summary of binding of human factor H to murine C3b. SE: standard error. R : response units. χ^2 : chi².

4.5.4 Decay acceleration assay

4.5.4.1 Murine Factor H does not accelerate the decay of the human C3 convertase

In the alternative pathway of complement activation, cleavage of C3 continuously generates C3b, which either remains in the fluid phase or binds covalently to nearby surfaces. Proenzyme factor B (FB) can bind to C3b, whereupon FB is cleaved by factor D (FD) to form C3bBb. C3bBb cleaves further molecules of C3 to C3b. Numbers of both fluid-phase and surface-bound C3b molecules are therefore rapidly amplified *via* a positive feedback loop unless this process is arrested by regulators. FH, the principle soluble regulator of complement, can suppress C3b amplification by accelerating the decay of C3bBb (206).

In Figure 4.16, C3b was amine coupled to a CM5 chip then FB and FD were flowed over this surface in the SPR instrument forming C3bBb that decays over a timescale of minutes. Recombinant mFH was then injected and the

acceleration of decay was observed. For reasons of availability, human C3b, FB and FD were used for this initial assay of decay-accelerating activity (DAA).

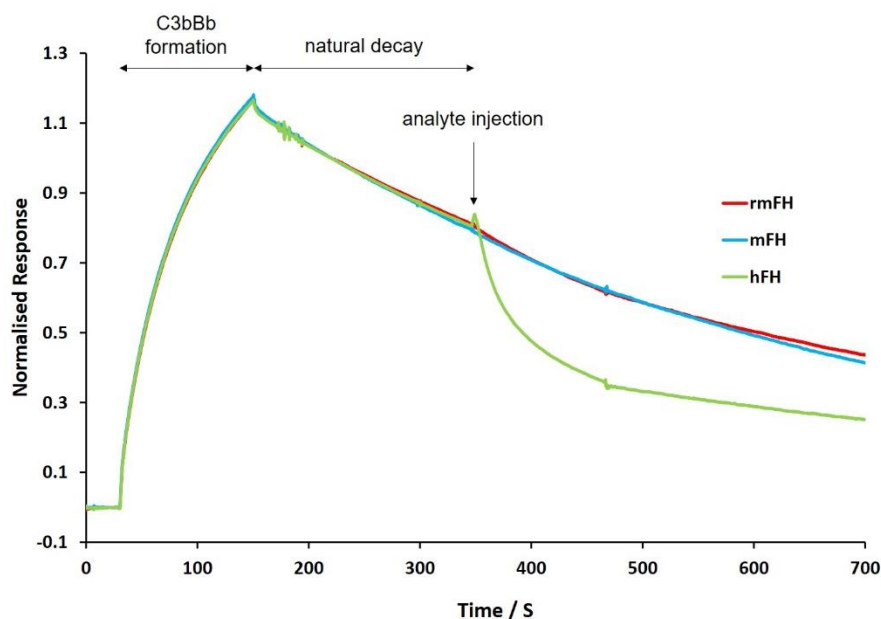


Figure 4.16 Decay-acceleration assay of human C3bBb by murine FH versus human FH. Human C3bBb (formed from human C3b, human FD and human FB) initially begins to dissociate (decay) to yield C3b and Bb due to its inherent instability. Following injection of 100nm rhFH the rate of decay is augmented. No such effect is observed with either of the two (recombinant or serum-derived) murine FH (100 nM) samples.

Figure 4.16 shows that neither recombinant nor native murine FH accelerates the decay of human C3bBb complex. This is despite murine FH binding to immobilised human C3b (on the SPR chip) with an affinity resembling its binding to murine C3b (Sections 4.5.1 and 4.5.2). This result is consistent with the hypothesis that FH catalyses C3bBb decay by binding simultaneously to *both* C3b and Bb within the transition state for the process whereby they dissociate, thereby stabilising this state and lowering the kinetic barrier to decay. According to this notion, mouse FH can bind human C3b but not human FBb.

4.5.4.2 Murine factor B is required to form the C3 convertase with murine C3b

Murine C3b and FB were gifts from Professor Santiago Rodriguez de Cordoba. Figure 4.17 shows the C3bBb convertases formed by using a combination of mC3b/hC3b and hFB/mFB. Human FD was used in both of these experiments.

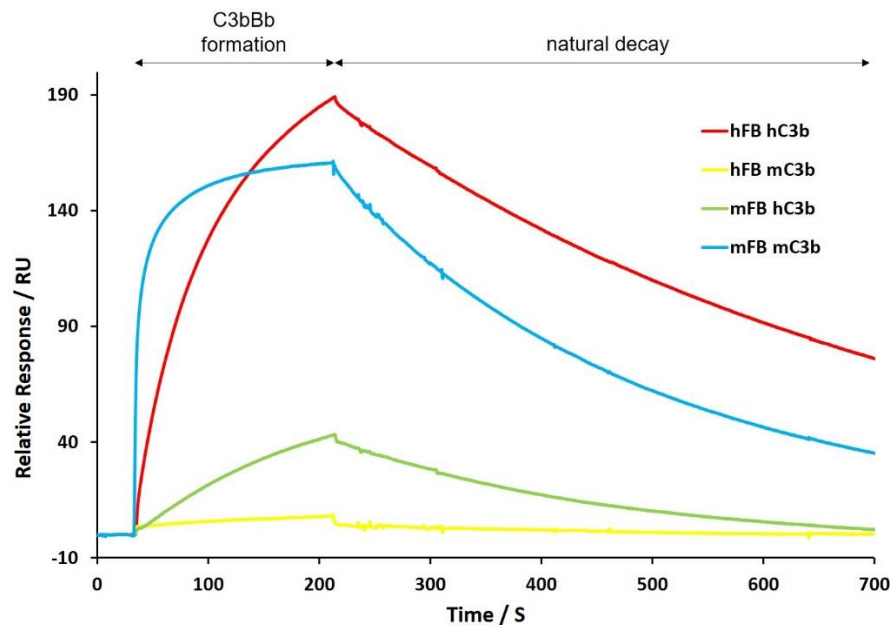


Figure 4.17 Formation of C3bBb hybrid convertases. Convertases formed using human or murine C3b and human or murine factor B.

Figure 4.17 indicates that complexes form when using hC3b/hFB or mC3b/mFB as expected. Much less convertase is formed between mouse FB and human C3b while human FB and mouse C3b fail to form any detectable complex. Note that it was previously shown using SPR assays that C3b interacts with FB but not with FD (11).

One interpretation of these results is that heterologous C3b and FB interact poorly if at all. Another interpretation is that while they can bind transiently to one another, this does not lead to mature complex formation. The published crystal structure of free FB shows a “locked” conformation in which the scissile bond (Arg²³⁴–Lys²³⁵) is protected from FD cleavage. It is thought that the binding of C3b to FB induces a conformational change that exposes the

scissile bond for cleavage by FD and subsequent formation of Bb and the convertase (207). Thus binding of either hC3b to mFB or mC3b to hFB, may not allow such a conformational change to fully take place. However, we cannot definitively distinguish between the C3bBb complex and the C3bB complex in this assay (no control was run without FD) so it is also possible that even in the case of mFB/mC3b no Bb is in fact produced by hFD action.

Figure 4.16 showed that mFH is unable to speed up the decay of human C3bBb complex. Figure 4.18 shows however that hFH is able to decay the murine C3bBb complex. This finding is consistent with the successful use of hFH as a complement regulator in murine *in vivo* experiments (83, 139).

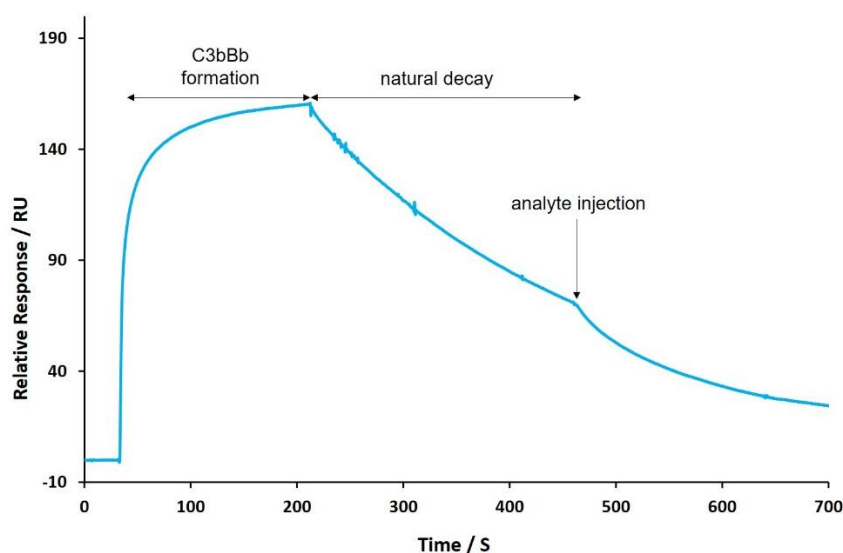


Figure 4.18 Decay acceleration of murine C3bBb by human factor H. Murine C3bBb is formed and undergoes natural decay, followed by an injection of 40 nM hFH.

4.5.4.3 Recombinant murine factor H acts as a decay accelerator for the murine C3 convertase

A recombinant version of human FH was shown to act as a decay accelerator for human C3 convertase, both as a full-length native-sequence protein (23) and in a truncated 'mini-FH' version (208). Both these molecules enhanced decay acceleration compared to serum-purified hFH at the same concentration. In the case of full-length rhFH, this was postulated to be due to

the lack of N-glycans and hence a higher proportion of molecules with an “activated conformation” compared to the serum-purified FH preparation.

Figure 4.19 shows the action of rmFH, rmFH-CHA and mFH on the murine C3 convertase (formed with human FD). All three murine FH proteins accelerate the decay of murine C3bBb. As was observed for the human orthologues, rmFH (and rmFH-CHA) have higher decay acceleration activity than mFH purified from serum. There is no discernible difference in the decay acceleration of rmFH (*i.e.* including clipped material) compared to rmFH-CHA (*i.e.* free of clipped material).

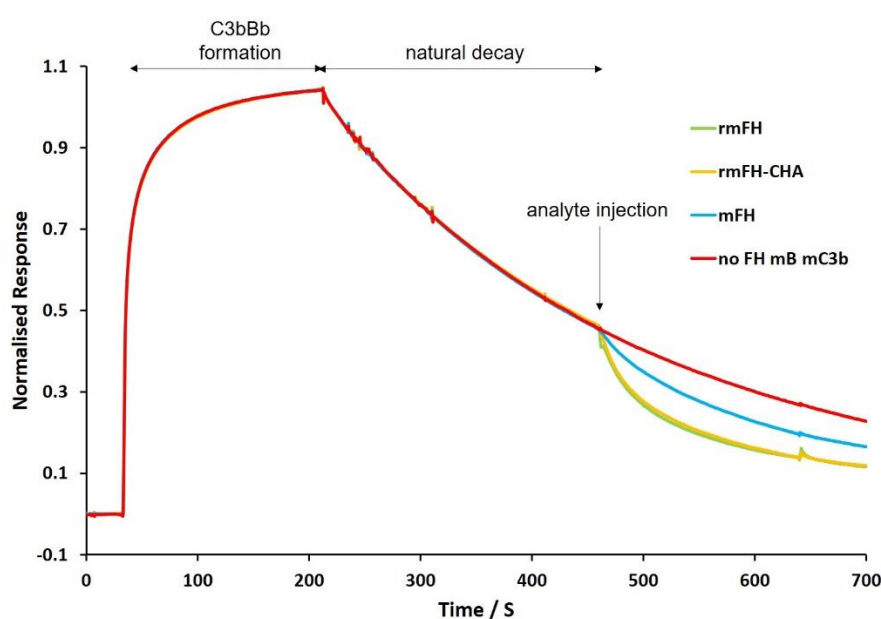


Figure 4.19 Murine C3bBb decay is accelerated by murine factor H. Murine C3bBb is formed and undergoes intrinsic decay. The rate of decay is accelerated following injection over the chip of recombinant proteins, rmFH and rmFH-CHA, or of serum-derived mFH (all at 40 nM).

4.6 Discussion

The data presented in this chapter show that a recombinant version of mouse FH, manufactured from a codon-optimised gene in the yeast *P. pastoris*, has the characteristics expected of a biologically functional protein. It binds to murine C3b, the key activation-specific fragment of C3, and it accelerates the decay of the murine C3 convertase (C3bBb) as effectively as native mFH.

Indeed, recombinant mFH has slightly more decay-accelerating activity than native mFH. Samples of recombinant mFH may contain a larger proportion of functionally active molecules than mouse serum-purified FH.

A similar observation was made for human orthologues (23, 208). It was suggested that the N-glycans of native FH stabilise a less active conformation that only becomes fully active when needed e.g. at self-surfaces requiring protection from complement activation. These surfaces are rich in sialic acid-terminated N-glycans and it has been postulated that these compete with FH-attached sialic acids for binding to sialic acid-binding sites on the FH molecule to bring about activating conformational changes.

It should also be borne in mind that serum-purified material is pooled from multiple individual animals, containing polymorphic variants. Moreover the FH molecules could have been circulating for days and hence could be damaged by glycation, nitration, oxidation or acetylation (23). The DLS data for serum-derived mFH suggested the presence of some aggregated material, while the SPR traces for mFH binding to immobilised C3b included anomalies and gave low maximal responses. Some of the recombinant mFH also sustained some damage due to cleavage in CCP 15 (creating fragments held together by a disulfide) but this did not seem to impact on its activities.

Hence, if the goal is homogeneous and maximally active FH, heterologous DNA expression followed by deglycosylation may afford advantages over serum-purified material. A disadvantage of deglycosylation is that the recombinant protein is less soluble although it did not show much propensity to self-associate at the concentrations used in these studies. The effects of lacking glycosylation will be further examined *in vivo* (Chapter 6).

There is a limited amount of *in vitro* data in the literature regarding the binding affinities of mouse FH for mouse C3b/d. In this Chapter, recombinant murine FH and its C3-derived targets were shown to interact about as tightly as their human orthologues. Moreover, rmFH binds human C3b and mouse C3b with similar affinities. It does not, however, accelerate the decay of the human C3

convertase of the alternative pathway (C3bBb), presumably because it does not bind so well to the Bb component (which is probably a requirement of decay acceleration). Conversely, hFH is able to decay the equivalent murine C3 convertase, which is of note given previous *in vivo* work in which hFH has been administered to CFH^{-/-} mice. As a side-product of the functional studies in this Chapter, it emerged that a hybrid C3bBb complex can be formed by mixing murine factor B, human C3b and human factor D but no detectable quantities of hybrid complex could be formed by mixing human factor B, murine C3b and human factor D. It would be interesting to study the catalytic properties of the hybrid hC3b.mBb convertase and its ability to cleave human and mouse C3.

Encouragingly, the sample that contained some clipped molecules (termed rmFH in this Chapter) yielded results in SPR-based assays that were almost identical to those of rmFH-CHA (a scarcer sample from which clipped material had been removed). The much more plentiful stock of rmFH was therefore subsequently used for *in vivo* studies (Chapter 6).

5: Functional characterisation of recombinant human factor H variants

5.1 Augmentation of factor H by a bacterial protein

Experiments in this section were performed in conjunction with Dr Andrew Herbert (School of Chemistry) (and published in a co-authored paper, (160)) using V62-rhFH and D1119G-rhFH produced by Dr Heather Kerr in Chapter 3 (3.3.1, 3.3.2).

The cleavage of complement protein C3 to C3a and C3b promotes inflammation. The amplification of C3b production *via* the alternative pathway of complement activation is driven by a positive-feedback loop that, unless regulated, leads to consumption of fluid-phase C3 and to indiscriminate coating of all nearby surfaces with C3b *via* a covalent linkage mediated by its thioester domain (TED). Sequential, further cleavage of C3b yields iC3b then C3dg and finally C3d. The C3d fragment equates to the TED and it remains attached to the cell surface long-term where it enhances adaptive immunity.

FH is a potent regulator of C3b production that works preferentially on self-surfaces. This specificity is thought to be mediated by glycosaminoglycans (GAG), and in particular by sialic acid (SA)-terminated glycans (209), present on host cells. Several GAG/SA-binding sites are present in FH including a region of CCPs 19-20 that interacts both with SA and the TED within C3b, iC3b and C3d (24).

The importance of FH as a complement regulator is highlighted by numerous reports that mutations in FH are associated with disease (210). Multiple different mutations in FH CCPs 19-20 are, in particular, associated with atypical haemolytic uraemic syndrome (94, 211). One such mutation, D1119G, when characterized in the context of a recombinant FH fragment comprised of CCPs 19 and 20, markedly reduces both C3b and C3d binding compared to wild-type FH (16, 99).

In an attempt to evade destruction by the complement system, many microbes bind and potentially capture circulating FH molecules (62, 212). The protein PspC, from the bacterium *Streptococcus pneumoniae*, is a good example. It recruits host-derived FH from plasma and traps it on the bacterial surface (213, 214) as a means of avoiding attack by the complement system. The following experiments were carried out to investigate the binding of the N-terminal domain of the D39 variant of *S. pneumoniae* (henceforth denoted as PspCN) to FH. They also explored the effects that binding to PspCN has on the binding of FH to C3b and C3d. Finally they examined the functional consequences of PspCN binding on both full-length rhFH and D1119G-rhFH.

5.1.1 PspCN binds virtually irreversibly to factor H

Figure 5.1 shows data from an SPR measurement. Here, a two-fold dilution series, from 8 to 0.25 nM, of either plasma-purified hFH (CompTech), recombinant human FH (rhFH) or D1119G-rhFH, was flowed (in duplicate) over a D39 His-tagged SUMO fusion version of PspCN immobilized (to an NTA chip). The binding is extremely tight for all three FH proteins with an off-rate that is too slow to measure (Figure 5.1d)

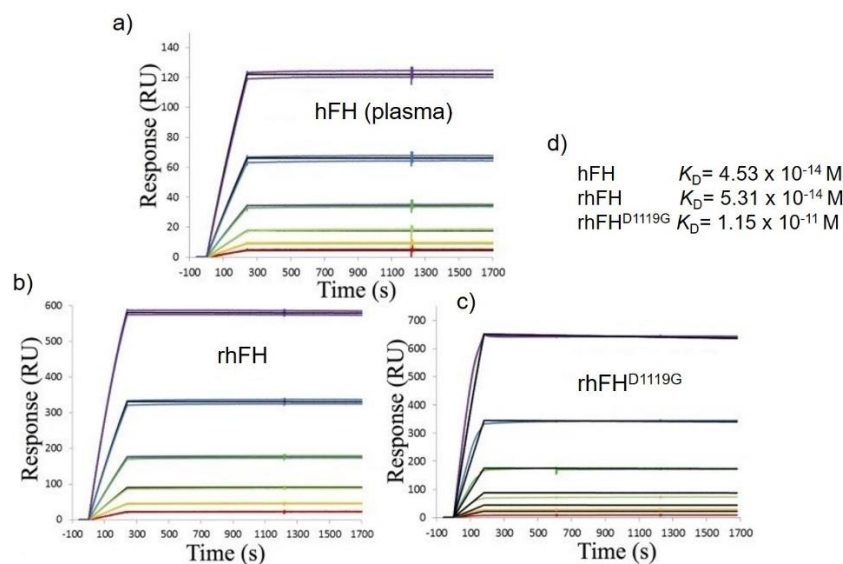


Figure 5.1 Factor H binding to PspCN. Two-fold dilution series of FH flowed over His-SUMO-PspCN immobilized to an NTA SPR chip. a) plasma-purified hFH; b) recombinant hFH; c) D1119G-rhFH. d) Binding affinity for each FH protein to PspCN. Solid black lines indicate fitted curves. Figure adapted from (160).

Further binding studies (Figure 3 in Herbert et al (160)) indicated that PspCN binds primarily to the central CCPs of FH (CCPs 8-10) but that full-length FH is required to achieve the binding affinities seen in Figure 5.1. Mass spectroscopy combined with cross-linking analysis suggested that PspCN binding brings about a conformational change in full-length FH. The PspCN-stabilised conformation of FH is thought to mediate its enhanced binding to C3b and C3d.

5.1.2 Binding of PspCN to full-length factor H exposes a new binding site for the thioester domain/C3d

Human C3b and C3d were amine coupled to a C1 SPR chip as previously described (2.7.4.2). Solutions of plasma-purified human FH (pre-incubated with or without a two-fold molar excess of PspCN) were injected over the chip. Figure 5.2 shows the sensorgrams and affinity curves for binding to C3b/d of hFH with or without PspCN. From these data, the K_D for hFH binding to C3b was calculated as 0.49 μM , which is consistent with values reported in the literature (161). Notably, the FH:PspCN complex was found to bind more tightly than FH alone to C3b, with an estimated K_D of 0.16 μM (Figure 5.2 a and b).

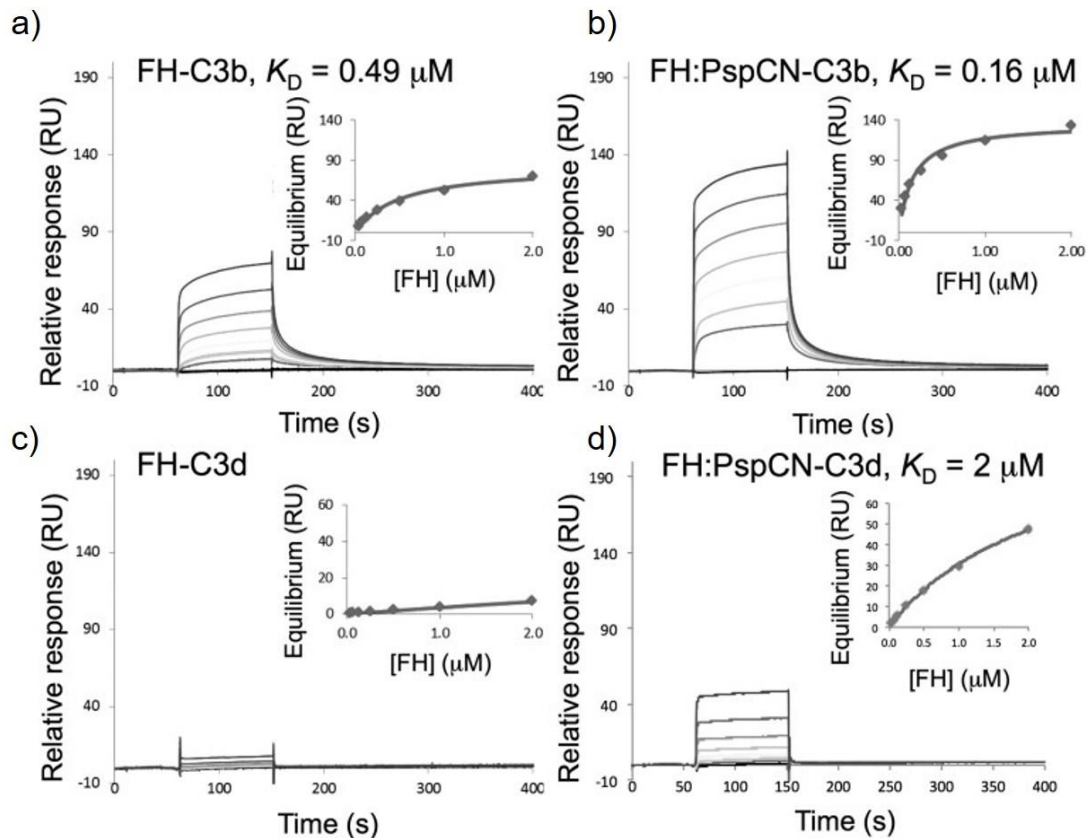


Figure 5.2 Plasma-purified factor H with/without PspCN binding to C3b/d. Background-subtracted sensorgrams with inset figures showing plot of response units *versus* concentration. a) hFH binding to C3b b) hFH:PspCN binding to C3b c) hFH binding to C3d d) hFH:PspCN binding to C3d. Figure adapted from (160).

In keeping with previous studies (16, 208) full-length plasma-purified hFH bound very poorly to immobilised C3d (Figure 5.2 c). Conversely, a truncated CCP 19-20 hFH fragment was shown to bind immobilised C3d with an affinity of $\sim 2 \mu\text{M}$. Figure 5.2 d) shows that, remarkably, hFH:PspCN binds C3d with an estimated affinity of $2 \mu\text{M}$, which is very similar to the affinity for C3d of the CCP 19-20 fragment. It was previously hypothesised that the C3d-binding site of hFH is inaccessible within the context of full-length hFH when hFH is operating on a foreign surface such as a bacterium or an SPR chip. These new data imply that binding of PspCN to full-length hFH re-models hFH and exposes the C3d/TED-binding site. This allows the PspCN:hFH complex to bind well to C3d, and more tightly to C3b than hFH alone (*i.e.* using two sites – CCPs 1-4 and CCPs 19-20, rather than just CCPs 1-4). This ‘more active’

form of hFH could be similar to that induced by markers on self-surfaces requiring protection from complement activation.

The above experiment was repeated with recombinant (r)hFH in lieu of plasma-purified hFH; Table 5.1 shows the collated binding affinities for C3b, and Table 5.2 shows the equivalent values for C3d.

	K_D (μM)	SE (μM)	R_{max}	χ^2
hFH	0.49	0.024	76	0.14
hFH + PspCN	0.16	0.059	134	0.50
rhFH	0.60	0.053	147	16.0
rhFH + PspCN	0.28	0.060	113	4.03

Table 5.1 Summary of binding of plasma-purified and recombinant (r) hFH +/- PspCN to C3b. SE: standard error. R : response units. χ^2 : chi². Table adapted from (160).

	K_D (μM)	SE (μM)	R_{max}	χ^2
hFH	ND	-	-	-
hFH + PspCN	2.3	0.16	102	0.40
rhFH	ND	-	-	-
rhFH + PspCN	2.2	0.37	100	0.8

Table 5.2 Summary of binding of plasma-purified and rhFH +/- PspCN to C3d. SE: standard error. R : response units. χ^2 : chi². ND: not detected. Table adapted from (160).

5.1.3 PspCN enhances complement-regulating function of FH

It was important to examine whether the PspCN-augmented binding of hFH to C3b and C3d enhances FH's important role as a regulator of the C3b-amplification loop. Figure 5.3 shows the effects of PspCN on the decay-accelerating activity (DAA) of plasma-purified hFH. As expected, 5 and 10 nM FH show dose-dependent enhancements of the decay of the C3bBb complex (the alternative pathway C3 convertase). In the absence of FH, 100 nM PspCN

has no effect on DAA. Notably, 5 nM hFH with a 2:1 ratio of PspCN was significantly more active than both 5 nM hFH and 10 nM hFH in the absence of PspCN.

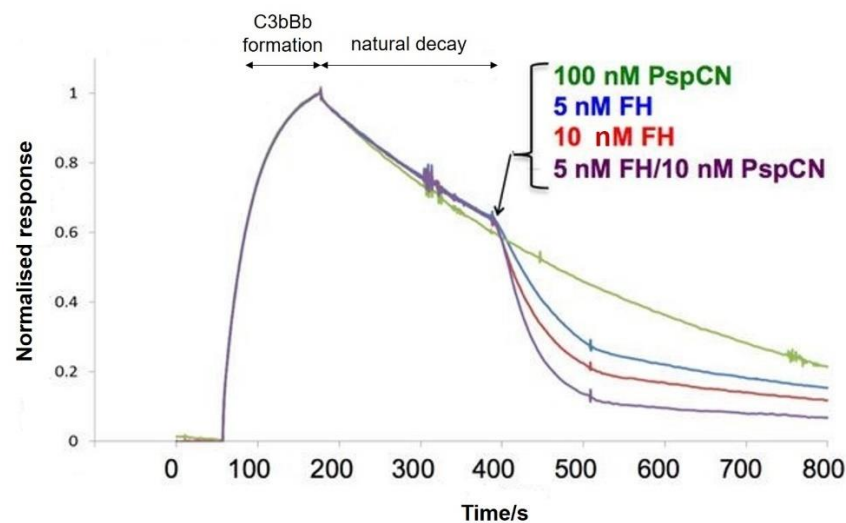


Figure 5.3 Decay-acceleration assay of C3bBb by factor H +/- PspCN. Human C3bBb (formed from human C3b, human FD and human FB) initially begins to dissociate (decay) to release Bb due to its inherent instability. Following injection of hFH +/- PspCN the rate of decay is augmented. No such effect is seen with PspCN alone. Figure adapted from (160).

Conversely, PspCN was found not to enhance the activity of plasma-purified hFH in a fluid-phase assay of factor I co-factor activity (for cleavage of C3b to iC3b) (160). Thus despite the observation that PspCN binding to hFH increases its affinity for C3b and boosts its DAA, it appears not to make hFH a better co-factor for factor I. One explanation could be that co-factor efficiency is limited by factor I recruitment, rather than by the affinity of hFH for C3b.

5.1.4 PspCN reveals effect of disease-linked factor H mutation D1119G

Results thus far imply that the C-terminus of full-length FH does not engage with C3b on a foreign surface. They suggest the TED/C3d-binding site remains occluded in the absence of PspCN or of molecular markers of a self-surface such as SA or GAGs. To test this hypothesis, rhFH with the aHUS-linked substitution D1119G was produced. This mutation has previously only been tested in the context of a CCP 19-20 fragment (16). In CCP 19-20, D1119G

markedly reduced both C3b and C3d binding. A crystal structure of FH 19-20 showed that Asp-1119 occupies a key position at the intermolecular interface.

Figure 5.4 a) shows that full length D1119G-rhFH binds to amine coupled C3b as tightly as plasma-purified “wild-type” hFH. Given that D1119G-rhFH presumably has a defective C-terminal TED/C3d binding site, this result lends weight to the hypothesis that the N-terminus of FH dominates its interactions on the SPR chip and hence presumably on any non self-surface. Note that D1119G-rhFH binds virtually irreversibly to PspCN (Figure 5.1 c) and it does not bind C3d (Figure 5.4 c).

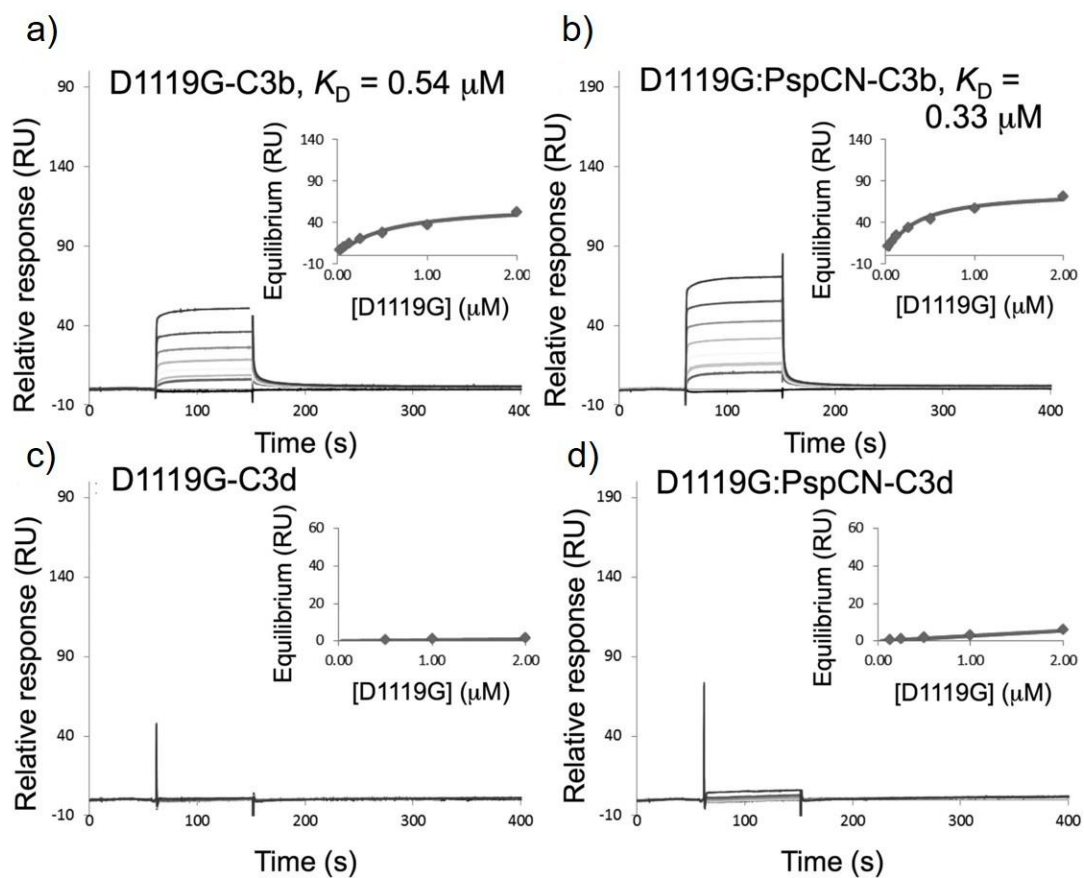


Figure 5.4 Binding of D1119G-rhFH, with or without PspCN, to C3b/d. Background-subtracted sensorgrams with inset figures showing plot of response *versus* concentration. a) the D1119G mutant of rhFH binding to C3b b) The D1119G mutant:PspCN complex binding to C3b c) the D1119G mutant binding to C3d d) The D1119G mutant:PspCN complex binding to C3d. Figure adapted from (160).

Figure 5.4 b) and d) show the results of D1119G-rhFH:PspCN complex binding to C3b and C3d respectively. In these experiments, PspCN appeared not to enhance the binding of D1119G-rhFH to C3d (K_D could not be calculated). This would be expected were PspCN to enhance binding of wild-type protein to C3b by exposing the C-terminal binding site, which is defective in the case of D1119G-rhFH. PspCN binding slightly improved the binding of D1119G-rhFH to C3b (0.54 μ M to 0.33 μ M). Such an enhancement would not be expected and if it were to be confirmed by further experiments (and for natively N-glycosylated FH) it might suggest that the C-terminus of hFH can still contribute a little towards bivalent binding even if the mutated C-terminus has a lower affinity.

Figure 5.5 shows the D1119G-rhFH mutation does not have a deleterious effect on the DAA of hFH (in the context of an SPR chip). This is as expected given the near wild-type binding affinity to C3b of D1119G-rhFH. The addition of PspCN caused a small improvement in the DAA of D1119G-rhFH, in contrast to the marked effect seen with wild-type hFH. This supports the hypothesis that the PspCN-induced improvement of DAA results from exposure of a C-terminal C3b/d binding site.

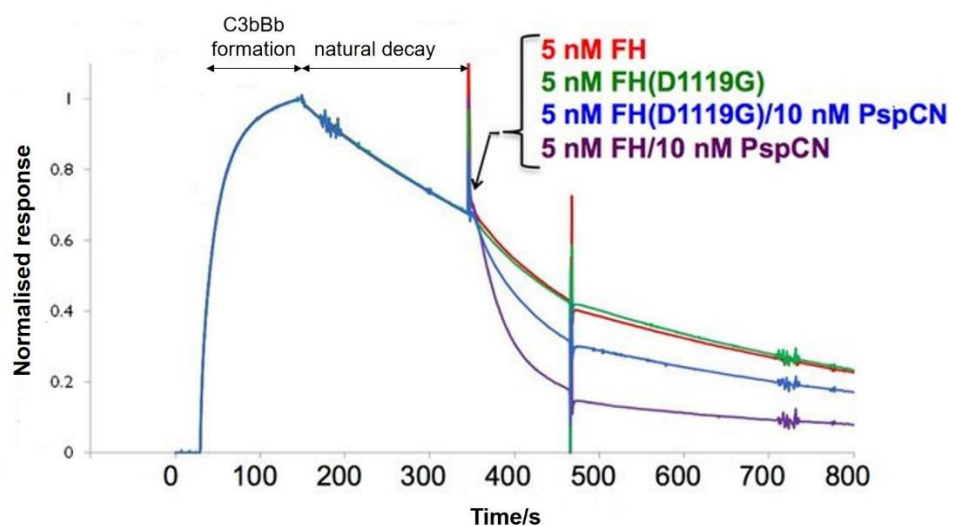


Figure 5.5 Decay-acceleration assay of C3bBb by factor H D1119G mutation +/- PspCN. 5nM D1119G-rhFH and 5 nM hFH have similar DAAs, but PspCN enhances the activity of wild-type hFH more than D1119G-rhFH. Figure adapted from (160).

The experiments described above focussed on the effect of PspCN on plasma-purified and recombinant hFH acting on what is in effect a foreign surface *i.e.* an SPR chip. To obtain data for a more physiological setting, haemolysis assays were performed using rabbit and sheep erythrocytes as surrogate host-cell surfaces. Rabbit erythrocytes lack human-like sialic acids, whereas sheep erythrocytes are rich in human like-sialic acids. Figure 5.6 a) shows that, as expected, the addition of wild-type hFH (purified from human plasma) to factor H-depleted normal human serum (FHΔNHS) did not afford significant protection against complement-mediated haemolysis to rabbit erythrocytes. Conversely, hFH protected sheep erythrocytes, displaying human-like N-glycans, from complement-mediated injury (Figure 5.6 b). Recombinant D1119G-rhFH protected neither rabbit nor sheep erythrocytes. Therefore, whilst the hFH mutant D1119G binds to C3b (on an SPR chip) as tightly, and decays C3bBb as efficiently, as wild-type hFH, it functions significantly less well on a surrogate host-cell surface. This, again, agrees with the hypothesis that FH binding to C3b on self-surfaces (*i.e.* as opposed to a “foreign” SPR-chip surface) induces exposure of the C-terminal TED/C3d binding site of wild-type hFH that is lacking in D1119G-rhFH.

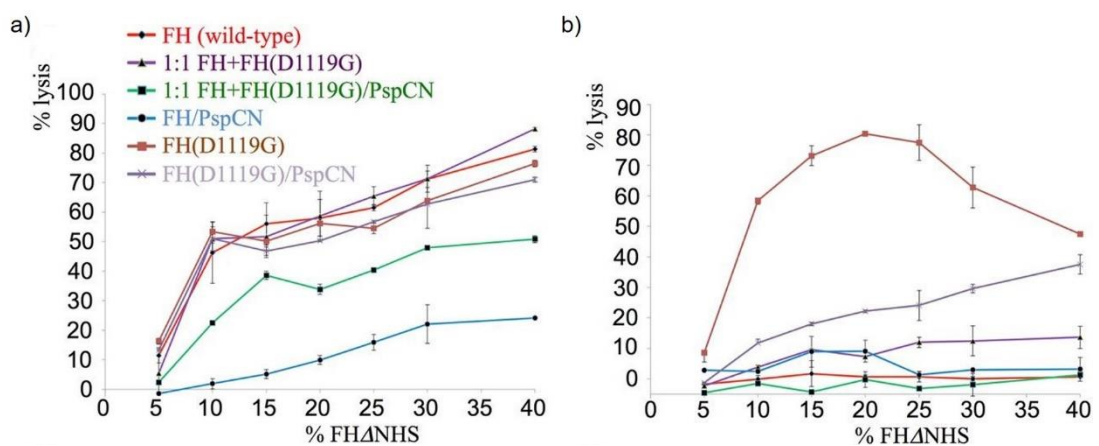


Figure 5.6 Erythrocyte-protection assays. a) Rabbit erythrocytes (surrogate foreign surface) b) Sheep erythrocytes (surrogate self-surface). D1119G-rhFH provides less protection to sheep erythrocytes than wild-type FH. Protection is enhanced by the addition of PspCN. Lysis measured by A_{412} of the released haemoglobin at 40% (v/v) FHΔNHS. Figure adapted from (160). 2μM FH added to FH-depleted serum.

5.1.5 PspCN can improve complement regulatory deficiency

In further erythrocyte-protection assays, mixtures of hFH and PspCN or D1119G-rhFH and PspCN were analysed for their abilities to regulate complement (Figure 5.6) The hFH:PspCN complex, (1 μ M hFH:2 μ M PspCN) compared to hFH alone, resulted in less haemolysis of rabbit erythrocytes (with a surrogate foreign surface) but did not fully protect the cells at the concentration used. Were PspCN attached to the surface it may afford a higher degree of protection.

Individuals with rare FH mutations are almost always heterozygous for those mutations. To recreate this situation, the complement-regulating properties of a (1:1) mix of 500 nM plasma-purified hFH and 500 nM D1119G-rhFH was investigated in the sheep erythrocyte-protection assay. Figure 5.6 b) shows that this mixture of mutant and wild-type hFH did not fully protect human-like sheep erythrocytes from complement-mediated lysis (unlike the pure wild type FH at 1 μ M). However when PspCN (at a ratio of 2:1) was added to this mixture, no lysis could be detected indicating complete protection. Note that PspCN also improved the ability of D1119G-rhFH without wild-type FH to protect sheep erythrocytes. The ability of PspCN to partially rescue the activity of D1119G-rhFH is consistent with the small improvements afforded by PspCN in C3b-binding and DAA functions of D1119G-rhFH, and may reflect a minor contribution from residual C3d binding at the PspCN-exposed, mutated C-terminus.

In summary, PspCN in the context of PspC attached to the surface of a bacterium may capture or “hijack” hFH. This not only localises FH to the bacterial surface but also augments its C3b binding and DAA by stabilising an “activated” conformation of the hFH molecule in which the “cryptic” C-terminal C3b/d-binding site is exposed. In this way levels of C3b tethered to the bacterial surface are maintained below the threshold for run-away activation *via* the positive feedback loop of the alternative pathway of complement activation. The ability of PspCN to improve protection against haemolysis

provided by a 1:1 mixture of D1119G-rhFH and wild-type FH (in FH Δ NHS) suggests the therapeutic potential of a FH-augmenting protein such as PspCN.

The results are consistent with a model in which hFH circulates in two conformational states with different functionalities. In its “latent” state one of two C3b-binding sites is masked; this corresponds to the relatively poor inhibitor of complement activation that is resistant to being exploited by most bacteria for their own protection from complement activation. In its augmented conformation, stabilised by binding to molecular markers on self-surfaces (or specialised bacterial proteins that have evolved to mimic self-markers), hFH is a very potent complement regulator ensuring self-surfaces are permanently protected from complement-mediated injury.

5.2 Disease-linked mutations in factor H reveal pivotal role of co-factor activity in self-surface selective regulation of complement activation

Experiments in this section were performed in conjunction with others (named below) and were published in a co-authored publication (161). Proteins were produced as described in Chapter 3 (Sections 3.3.1-3.3.3). All FH proteins were produced by Dr Heather Kerr, excepting I62-rhFH (Dr Andrew Herbert).

As previously discussed, a wide variety of mutations in FH are linked to disease (94, 215). However, the penetrance for many of these mutations is low. Variations in the genes for other complement proteins and environmental factors are also likely to contribute to disease risk (134). To explore the functional effect of each disease-causing mutation using patient samples is difficult, as most patients are heterozygous for the mutation that in any case occurs on a polymorphic background, and the availability of pure FH samples is limiting (163).

Many studies have therefore tried to recapitulate disease-linked FH mutations in easily produced FH truncations e.g. CCPs 1-4 or CCPs 19-20, which is where most disease-causing mutations reside (37, 99, 203, 216, 217).

However these uses of truncated hFH do not fully capture the cooperation and collaboration that could occur between the multiple binding sites that are located throughout the length of this apparently elongated and flexible 20-module protein. Given previous success in production of full-length recombinant FH (Section 3.3.1) and characterisation of the FH mutation D1119G using the bacterial protein PspCN (Section 5.1.4), it was decided to produce and test additional mutations, as seen in Figure 5.7.

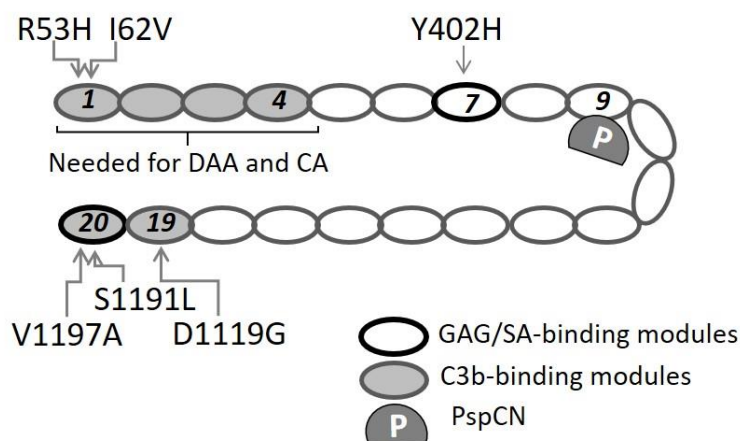


Figure 5.7 Schematic of factor H highlighting mutations used in this study. C3b-binding sites (shaded) are located in N- and C- terminal regions. Self-surface recognition sites, which bind to GAGs and SAs, reside in CCPs 7 and 20. The SNP Y402H is linked to age-related macular degeneration (AMD) and C3 Glomerulopathy (H402 is the at-risk SNP). PspCN binds to CCPs 8-10. Figure adapted from (161).

5.2.1 Comparison of wild-type factor H V62 and I62

The original full-length rhFH produced by our laboratory (23) was VYE-FH; *i.e.* it had Val at position 62, Tyr at position 402 and Glu at position 936. The presence of Ile 62 in hFH is protective for age-related macular degeneration (163). FH with His at 402 is a common variant linked to age-related macular degeneration (AMD) and C3 Glomerulopathy (149, 218). The common SNP corresponding to Glu at 936 of hFH confers susceptibility to meningococcal disease (152) – however this residue is not located near binding sites for C3b or carbohydrates and so is unlikely to affect any of the assays employed in the current work.

Previous work (203) compared rhFH CCP 1-4 fragments corresponding to Ile 62 *versus* Val 62 variants. Major differences between binding properties can occur according to context (e.g. rhFH CCP 19-20 binds C3d, while full-length rhFH does not). Hence, full-length rhFH containing Ile 62 (with Tyr at 402 and Asp at 936 *i.e.* I62-rhFH) was produced to compare with rhFH with Val at 62 *i.e.* V62-rhFH.

In the following experiments three FH proteins were investigated in SPR-based binding assays: plasma-purified hFH, recombinant V62-hFH and recombinant I62-hFH (denoted in subsequent figures as p-CFH, V62-CFH and I62-CFH respectively).

Similar K_D values were obtained for all three proteins when dilution series were flowed over C3b that had been amine coupled to a C1 chip. Values lay in the range 0.21-0.33 μM (Figure 5.8 a) and b), Table 5.3). C3d had been immobilised on a different flow cell of the chip (Figure 5.8 a) and c)). Both I62-rhFH and V62-rhFH bound to immobilized C3d with K_D values of $\sim 1.9 \mu\text{M}$ (Table 5.3). Plasma-purified FH did not bind to C3d, as previously reported (208)(Figure 5.2 c). This lack of binding to immobilised C3d of hFH from plasma has previously been attributed to lack of access to the TED/C3d-binding site in CCPs 19-20. Indeed, mixing 0.6 μM plasma hFH with 1.2 μM PspCN turned it into a C3d binder (Figure 5.8 d) as was seen before (Figure 5.2 d). Pre-incubating I62-rhFH 1:2 with PspCN, or V62-rhFH 1:2 with PspCN, also enhanced binding to C3d, as well as to C3b (Figure 5.8 d). For confirmation, two additional SPR-based experiments were performed on a different batch of I62-rhFH, on different days and with a fresh SPR C1 chip. In both experiments (Table 5.4), the K_D of I62-rhFH binding to C3b was approximately halved following pre-incubation with the two-fold molar excess of PspCN. It was concluded that plasma-derived hFH and both I62 and V62 variants of rhFH all bind to, and are augmented by, PspCN. They likely all undergo a similar conformational change (See Section 5.1.2) that in each case renders their C-terminal modules more accessible to C3d.

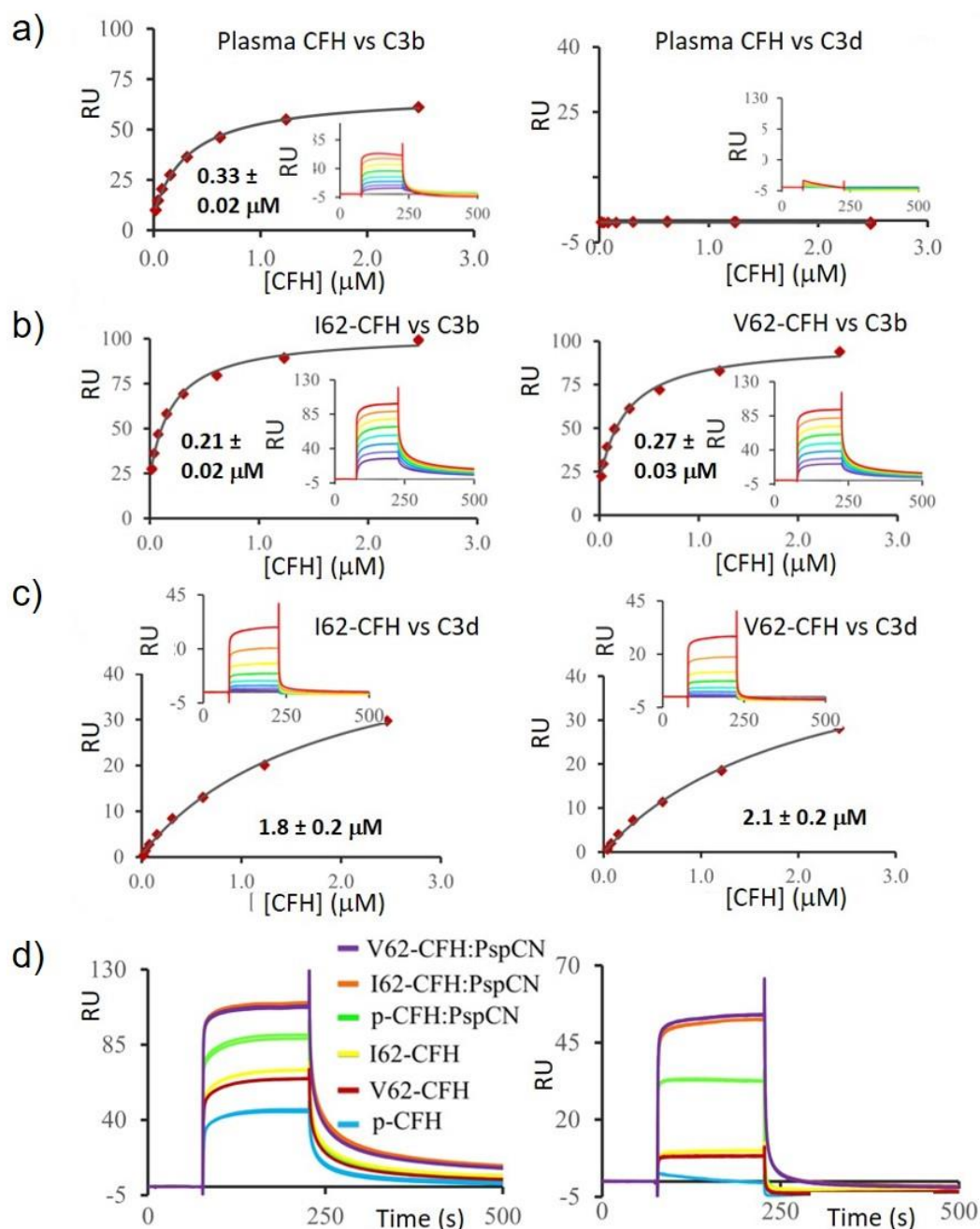


Figure 5.8 C3b and C3d binding of human plasma-derived FH, I62-(rh)CFH and V62-(rh)CFH. Experiment performed by Dr Eliza Makou (School of Chemistry). Data were collected, in a single SPR experiment, with C3b and C3d immobilised on different flow cells of the same C1 chip. Two-fold dilution series of hFH, from 2.5 to 0.019 μM , were injected over the chip. a)-c) show plots of response vs concentration, and background-subtracted sensorgrams shown in insets. a) Plasma FH binding to C3b (left) or C3d (right). b) I62-CFH binding to C3b (left) and V62-CFH binding to C3b (right). c) I62-CFH binding to C3d (left) and V62-CFH binding to C3d (right). Multiple replicates confirmed the difference in C3d binding between rhFH and plasma-derived FH. d) Sensorgrams for I62-CFH, V62-CFH or plasma FH (p-CFH), all at 0.6 μM , with or without PspCN (1.2 μM), flowed over C3b (left) or C3d (right). K_D values estimated for I62-CFH alone versus I62-CFH:PspCN (Table 5.3). Figure adapted from (161).

	Ligand	K_D (μM)	$S.E$ (μM)	R_{max}	χ^2
I62-rhFH	C3b	0.21	0.02	79.2	5.2
V62-rhFH	C3b	0.27	0.03	80.0	4.6
Plasma-hFH	C3b	0.33	0.02	59.6	0.6
I62-rhFH	C3d	1.8	0.02	50.6	0.3
V62-rhFH	C3d	2.1	0.02	52.2	0.2
Plasma-hFH	C3d	high	-	-	-

Table 5.3 Summary of binding of factor H to C3b and C3d. SE: standard error. R : response units. χ^2 : chi². Table adapted from (161).

The abilities of rhFH and plasma-derived hFH to accelerate decay of the alternative pathway C3 convertase (C3bBb) were also assessed in an SPR-based assay on a CM5 chip as previously described (Section 2.7.4.3). Both I62-rhFH and V62-rhFH had identical DAA in this assay, whereas plasma-derived hFH was less-active (Figure 5.9 a). A similar difference between plasma-derived FH and recombinant FH was also seen in the case of murine FH (Figure 4.19).

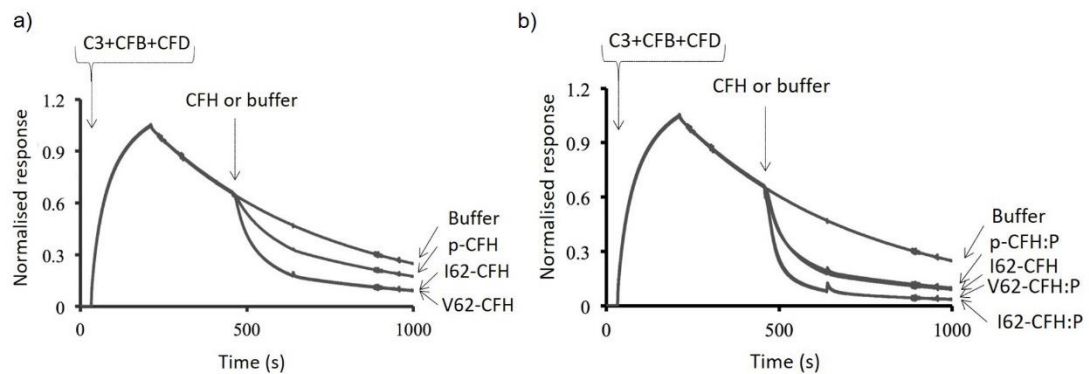


Figure 5.9 Decay accelerating activity of I62-(rh)CFH and V62-(rh)CFH. a) C3bBb was formed on a CM5-sensor chip by flowing factor B and D over immobilized C3b. The C3bBb DAA of 20 nM plasma-CFH, 20 nM I62-CFH and 20 nM V62-CFH were compared. b) The experiments in a) were repeated with a 1:2 mixture of FH and PspCN (i.e. CFH:P). The curve from a) for I62-CFH is included for reference. Note that I62-CFH and plasma-CFH:P give almost identical curves hence they are nearly superimposed. The data shown are representative of an experiment performed four times by Dr Eliza Makou. Figure adapted from (161).

Functional similarities between I62-rhFH and V62-rhFH were also seen in haemolysis-protection assays (see Section 5.1.4). Upon exposure to increasing percentages of FH-depleted normal human serum (NHS), sheep erythrocytes (E_S) are susceptible to lysis by complement unless protected by added FH. As discussed in Section 5.1.4, rabbit erythrocytes (E_R) lack human-like sialic acids and are not protected by FH. In these experiments, both I62-rhFH and V62-rhFH proved equally effective (at 250 nM) in preventing E_S haemolysis in this assay (Figure 5.10 a). As expected, both were equally ineffective (at 1 μ M) in protecting E_R from haemolysis (Figure 5.10b). Both I62-rhFH and V62-rhFH showed a greater ability to protect E_R from haemolysis when in complex with PspCN.

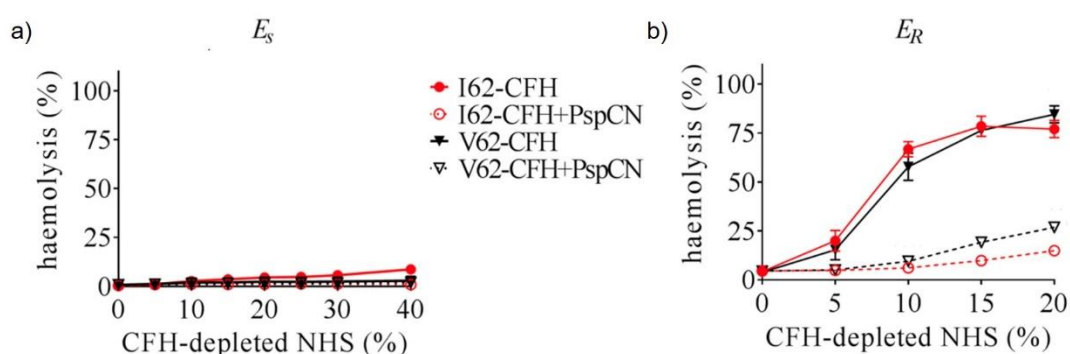


Figure 5.10. Erythrocyte-protection assays comparing I62-(rh)CFH and V62-(rh)CFH. E_S or E_R were incubated with CFH-depleted NHS at various percentages (v/v) that had been reconstituted with the rhFH variants. a) Reconstitution with 250 nM I62-rhFH or V62-rhFH afforded protection of E_S , with or without 500 nM PspCN. b) Neither 1 μ M I62-rhFH or V62-rhFH protected E_R . PspCN (2 μ M) enhances FH protection in both cases. Experiment performed by Dr Yi Yang (Institute of Cellular Medicine, Newcastle University). a) is representative of two separate experiments. Six independent measurements were made in b). The mean and S.D. (error bars) for each measurement were calculated for all data sets (error bars obscured by symbol). Figure adapted from (161).

In a more specific E_S -based cell-surface assay (163), C3bBb was pre-assembled on E_S and the resultant C3bBb-decorated cells were exposed to a FH concentration series. This was followed by an assay of surviving C3bBb, thus allowing estimation of an IC_{50} value for DAA on a 'host-like' cell surface. Both I62-rhFH and V62-rhFH exhibited an IC_{50} of ~ 1.3 nM in this assay (Figure 5.11 a), Table 5.5). In a related study, E_S surfaces were pre-loaded with C3b and then treated with FH and factor I (FI) in a concentration series. The

remaining C3b was estimated in a haemolysis assay as before. This provided an IC_{50} for the factor I co-factor activity (CA) of FH on a surrogate cell-surface. In this assay, both I62-rhFH ($IC_{50} = 8.0$ nM) and V62-rhFH ($IC_{50} = 9.5$ nM) had similar potencies (Figure 5.11 b) and Table 5.5). The effect of PspCN on both of these assays was explored for I62-rhFH. Unexpectedly, I62-rhFH (alone) and I62-rhFH:PspCN complex had indistinguishable DAAs towards E_S -bound C3bBb (Figure 5.11 c). Interestingly, PspCN improved the CA of I62-rhFH on E_S -bound C3b by a factor of 2 (Figure 5.11 d) and Table 5.5).

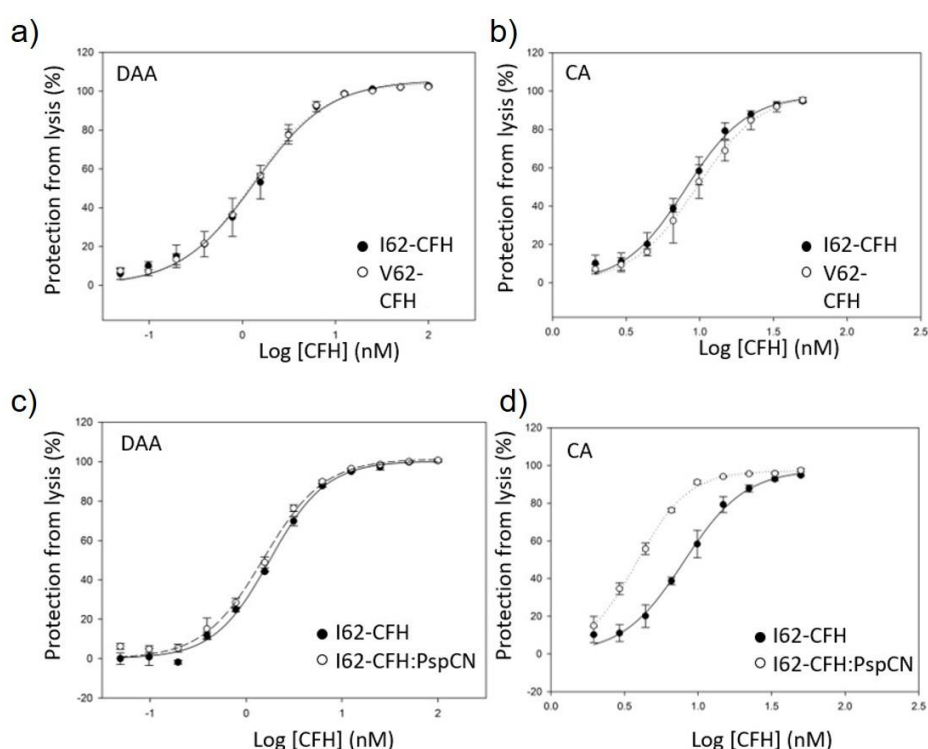


Figure 5.11 Decay-accelerating and co-factor activities of I62-(rh)CFH and V62-(rh)CFH on sheep erythrocytes. a) Activated sheep erythrocytes (E_S) were preloaded with C3bBb. The C3bBb-decorated cells were exposed to a FH-concentration series, followed by a haemolysis-based estimate of remaining C3bBb. This allowed derivation of IC_{50} values for DAA on a host-like cell surface. b) C3b-preloaded activated E_S surfaces were treated with factor I and a range of FH concentrations, providing an estimate of the CA of FH on a host-like cell surface. c) the DAA in a) was repeated, but using I62-CFH mixed 1:2 with PspCN. The curve for I62-CFH alone is included for comparison. d) the assay used in b) was repeated, but using I62-CFH premixed 1:2 with PspCN. The curve for I62-CFH alone is included for comparison. The mean and S.D. (error bars) of triplicate measurements are plotted, and sigmoidal curves were fitted using SigmaPlot. Each graph is representative of two experiments, except for a) and d), which show data from single experiments. Experiments performed by Dr Edwin Wong (Institutes of Genetic Medicine, University of Newcastle). Figure adapted from (161).

In summary, I62-rhFH and V62-rhFH bound with similar affinities to C3b and C3d on SPR chips. The two variants shared nearly identical DAAs on this man-made foreign surface. In haemolysis-protection assays, both I62-rhFH and V62-rhFH protected self-like E_S , but neither protected the less self-like E_R unless PspCN was present. PspCN enhanced binding of both I62-rhFH and V62-rhFH to C3b and C3d on SPR chips, where it also enhanced DAA. PspCN enhanced the CA, but not the DAA, of FH on E_S .

5.2.2 Functional characterisation of a disease-linked CCP 1 mutation

The disease-linked R53H mutation in FH CCP 1 was previously studied within the context of rhFH CCPs 1-4 (203). Although R53H did not disrupt the binding of C3b by FH, it did perturb both the CA and DAA of rhFH CCPs 1-4 on E_S . In the current work, these experiments were repeated with full-length R53H-rhFH (on an IYD-background). In side-by-side comparisons using SPR, R53H-rhFH bound about as tightly as I62-rhFH to amine-coupled C3b on a C1 chip, with estimated K_D values of 1.2 μM for I62-rhFH *versus* 1.8 μM for R53H-rhFH (Figure 5.12 and Table 5.4) in a first experiment and 1.0 and 1.2 μM for I62-rhFH and R53H-rhFH, respectively, in a second experiment (Table 5.4). Pre-incubation of R53H-rhFH with PspCN (in a 1:2 molar ratio) enhanced binding of C3b from 1.8 to 1.0 μM in the first experiment and from 1.0 to 0.6 μM in the second experiment. This PspCN-induced improvement in affinity is similar to that seen for I62-rhFH. As with the C3b binding studies in Chapter 4, all affinity constants are estimates due to the concentration range of FH used in the assays.

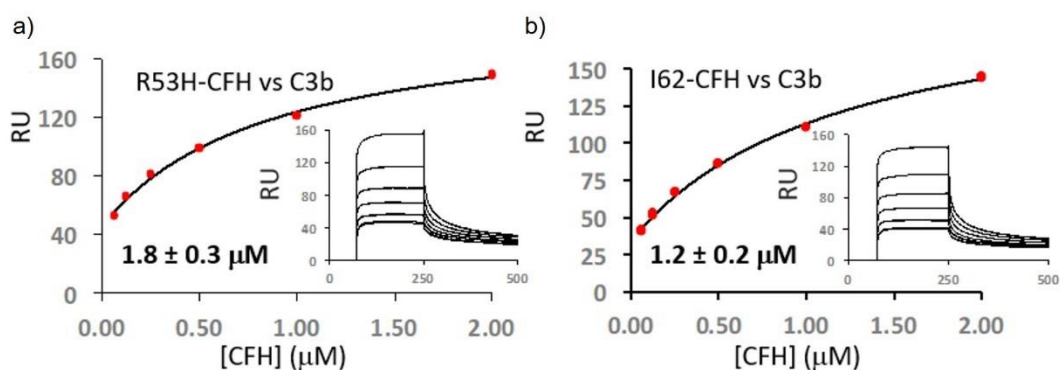


Figure 5.12 Effects of R53H-(rh)CFH on C3b binding. An SPR experiment was performed as previously described to compare the affinity for immobilized C3b of R53H-rhFH (a) with that of I62-(rh)CFH (b). Graphs show plots of response vs concentration, with background-subtracted sensorgrams in inset. Figure adapted from (161). Performed by Dr Heather Kerr.

Despite their similar affinities for C3b, 40 nM R53H-rhFH exhibited poorer DAA on a CM5-sensor chip (Figure 5.13) in comparison with 40 nM I62-rhFH. Similarly, in assays of DAA performed on C3bBb-decorated E_s , R53H-rhFH ($\text{IC}_{50} = 6.6 \text{ nM}$) performed less well than I62-rhFH ($\text{IC}_{50} = 1.4 \text{ nM}$) (Figure 5.14 a) and Table 5.5). In an assay on C3b-decorated E_s , R53H-rhFH ($\text{IC}_{50} = 43 \text{ nM}$) was likewise less effective in terms of its CA than I62-rhFH ($\text{IC}_{50} = 18 \text{ nM}$). PspCN (at a 2:1 molar ratio with FH) did not noticeably improve the DAA of R53H-rhFH measured on E_s (Figure 5.14 c), but PspCN did improve the CA of R53H-rhFH (Figure 5.14 d).

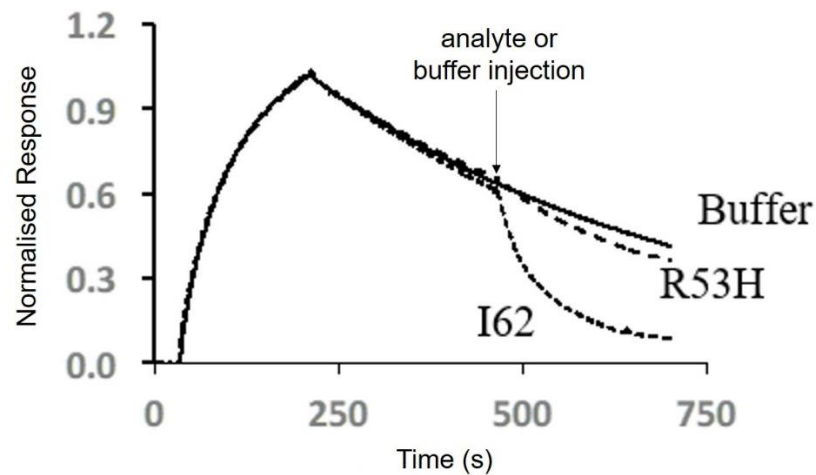


Figure 5.13 Effects of R53H-rhFH on decay acceleration assay. An SPR-based assay of DAA was performed as in Figure 5.9 except that 40 nM R53H-rhFH or 40 nM I62-rhFH were used. The data shown are representative of two experiments. Figure adapted from (161). Performed by Dr Heather Kerr.

The functional deficiencies of R53H-rhFH seen thus far were not immediately apparent in haemolysis-protection assays. R53H-rhFH with or without PspCN did not protect foreign E_R (Figure 5.15 b) as expected, but R53H-rhFH was able to protect the more 'self-like' E_S (Figure 5.15 a). Protection of E_S in this case may reflect the inability of R53H-rhFH to prevent turnover, and hence 'futile' depletion of, C3 in the fluid phase. To investigate this further, a 1:1 mixture of R53H-rhFH with I62-rhFH (resembling the physiological situation although using lower concentrations of FH) was tested in the haemolysis-protection assay. In this experiment, I62-rhFH might be expected to prevent fluid-phase C3 depletion. Protection of E_S was nonetheless observed (Figure 5.15 c), perhaps because R53H-rhFH antagonises and negates the fluid-phase C3-conserving regulatory action of I62-rhFH. Alternatively the I62-rhFH component of this 1:1 mixture might be adequate to achieve cell-surface complement regulation and protection.

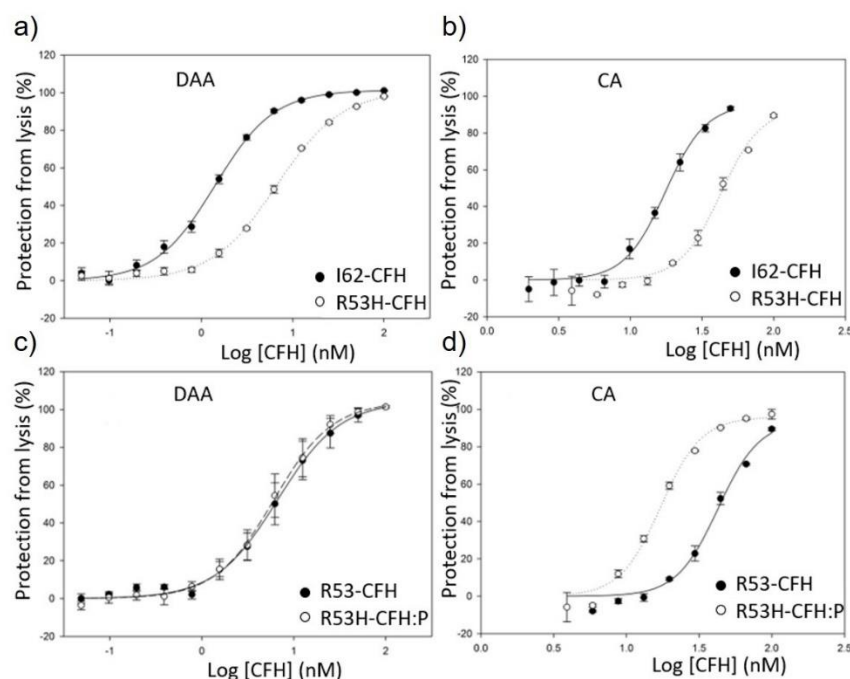


Figure 5.14 Decay-accelerating and co-factor activities of R53H-rhFH on sheep erythrocytes. a) An E_S -based assay of DAA was carried out in a similar manner to that shown in Figure 5.11 a). b) An E_S -based assay of CA was carried out in a similar manner to that shown in Figure 5.11 b). Triplicate measurements were made for each data point in a) and b). c) In an E_S -surface assay (as in Figure 5.11 a) PspCN (mixed 2:1 with R53H-rhFH) does not restore DAA to R53H-rhFH. d) In an E_S -surface assay (as Figure 5.11 b) PspCN (mixed 2:1 with R53H-rhFH) enhances its CA activity. The mean and S.D. (error bars) of triplicate measurements are plotted, and sigmoidal curves were fitted using SigmaPlot. Each graph is representative of two experiments. Experiments performed by Dr Edwin Wong. Figure adapted from (161).

In another experiment, R53H-rhFH was mixed in a 1:1 ratio with the S11191L/V1197A-FH (LA-rhFH) mutant. LA-rhFH prevents C3 depletion in the fluid-phase, although it exhibits impaired binding to sialic acid decorated surfaces (209). The R53H-rhFH:LA-rhFH mixture was less able to prevent E_S haemolysis than R53H-rhFH alone (Figure 5.15 c), consistent with non-depletion of C3, an expected consequence of the fluid-phase regulatory action of LA-rhFH. In summary, R53H-rhFH is functionally deficient despite retaining good affinity for C3b.

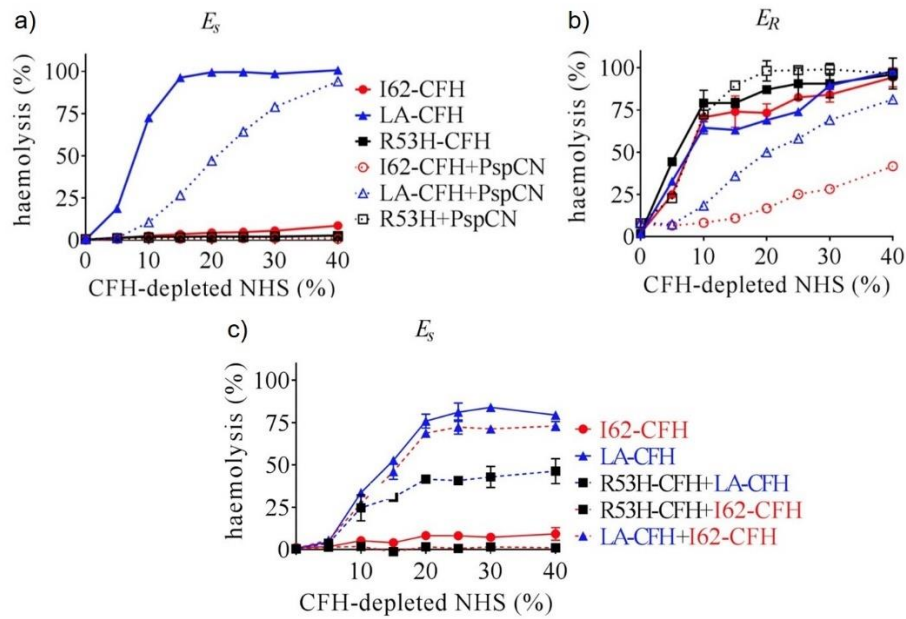


Figure 5.15 Erythrocyte haemolysis-protection assays for R53H-(rh)CFH and LA-(rh)CFH. a) LA-rhFH (500 nM) offered little protection against E_S lysis. No lysis occurred in the presence of 250 nM R53H-FH. The addition of 500 nM PspCN to 250 nM LA-rhFH improved its protective properties. b) Neither 1 μ M LA-rhFH (alone) or 1 μ M R53H-rhFH (with or without 2 μ M PspCN) protected E_R , but a mixture of 1 μ M LA-rhFH and 2 μ M PspCN was more protective than LA-rhFH alone. c) LA-rhFH (500 nM) did not protect E_S from lysis ;nor did a mixture of 250 nM LA-rhFH and 250 nM I62-CFH. A mixture of 250 nM R53H-rhFH and 250 nM I62-CFH was protective. A mixture of 250 nM R53H-rhFH and 250 nM LA-rhFH was more protective than 500 nM LA-rhFH alone but less protective than R53H-rhFH alone (see a). a) to c) are representative of two independent experiments performed by Dr Yi Yang. The mean and S.D. (error bars) for each measurement were calculated for all data sets (error bars obscured by symbol). Figure adapted from (161).

5.2.3 Perturbation of the C-terminal C3b/d-binding site

As discussed in 5.1.4, Asp-1119 of FH CCP 19 is a key interface residue in the FH 19-20:C3d complex (16). In the context of rhFH 19-20, D1119G abrogated binding to C3b and C3d (16, 99) and destroyed the ability to compete with plasma FH for binding to C3b-decorated E_S (37, 99). In Figure 5.6 D1119G appears to degrade performance of full-length rhFH in E_S haemolysis-protection assays. The DAA and CA of full-length D1119G-rhFH were measured on E_S . As shown in Figure 5.16 a) and Table 5.5, the ability of D1119G-rhFH to accelerate decay of C3bBb on E_S was diminished compared with I62-rhFH ($IC_{50} = 1.3$ nM for I62-rhFH versus $IC_{50} = 11$ nM for D1119G-rhFH). The negative effect of D1119G-rhFH on the CA of FH for cleavage of

C3b bound to E_S appears even greater ($IC_{50} = 12$ nM for I62-rhFH versus $IC_{50} > 500$ nM for D1119G-rhFH) (Figure 5.16 b). PspCN (at a 2:1 molar ratio to D1119G-rhFH) had negligible effects on the DAA (Figure 5.16 c) and CA of D1119G-rhFH, measured on E_S (Figure 5.16 d).

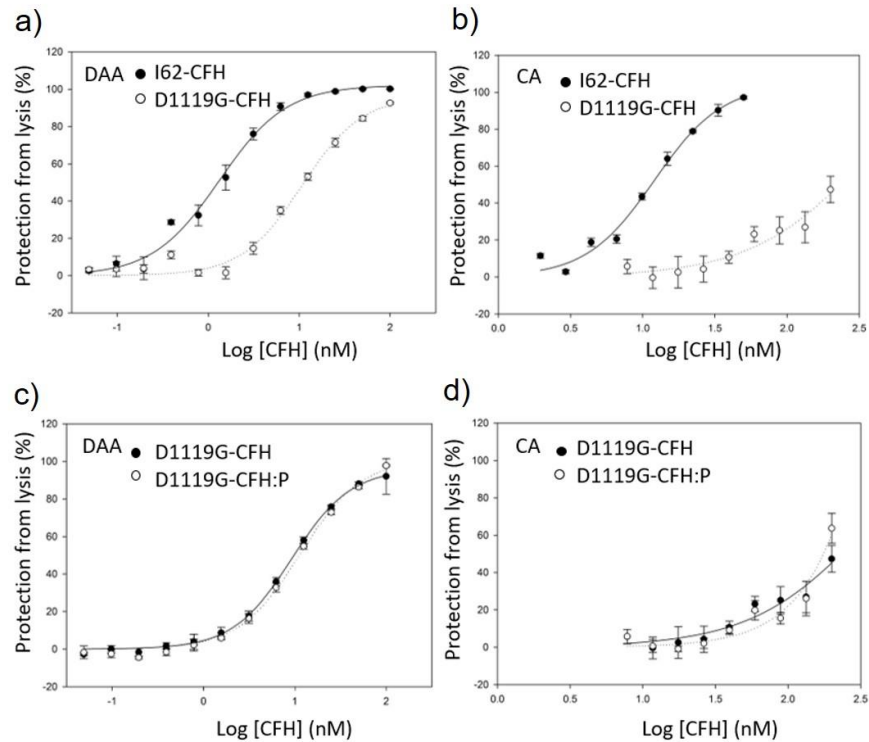


Figure 5.16 Effects of D1119G-rhFH on erythrocyte-based assays. In similar assays used to generate Figure 5.11 and 5.14, D1119G decreased the DAA and CA of rhFH on E_S (a and b). c) PspCN (mixed 2:1 with D1119G-rhFH) had little effect on the DAA of D1119G-rhFH measured on E_S . d) PspCN (mixed 2:1 with D1119G-rhFH) had a small effect on the very low CA of D1119G-rhFH measured on E_S . Mean and S.D. (error bars) of triplicate measurements are plotted, and sigmoidal curves fitted using SigmaPlot. Each graph is representative of two experiments performed by Dr Edwin Wong. Figure adapted from (161).

5.2.4 Perturbation of the sialic acid-binding site in hFH CCP 20

Ser-1191 and Val-1197 in CCP 20 are critical for recognition of sialic acids according to a crystal structure of a C3d:FH 19-20:sialic acid complex (209). In previous work, the disease-linked mutation S1191L/V1197A disrupted the ability of rhFH CCPs 19-20 to compete with full-length hFH for binding to C3b-decorated E_S (16, 99) but did not perturb the 3D structure of CCPs 19-20 nor interfere with their binding, measured by SPR, to immobilised C3b or C3d (217). In this study, we found that the SPR-measured affinities of full-length

LA-rhFH and I62-rhFH are similar: $K_D = 0.8 \mu\text{M}$ for LA-rhFH *versus* $1.2 \mu\text{M}$ in one experiment (Figure 5.17 and Table 5.4) and $0.9 \mu\text{M}$ for LA-rhFH *versus* $1.0 \mu\text{M}$ in the second experiment (Table 5.4).

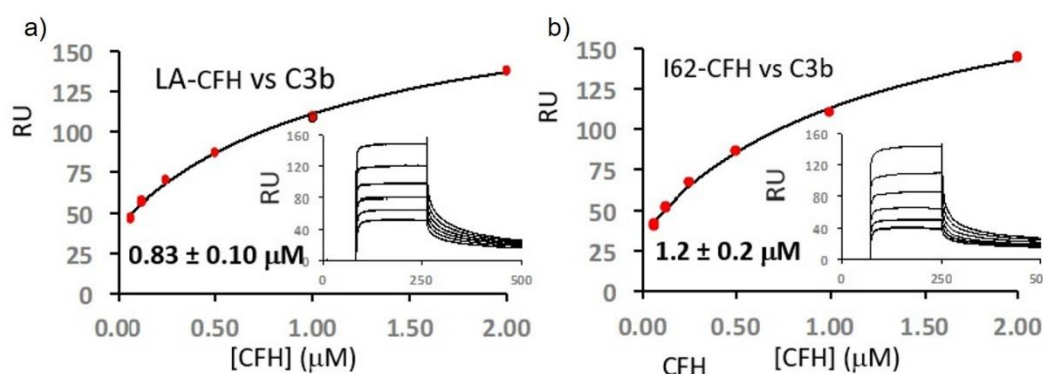


Figure 5.17 Effects of the S1191L/V1197A double substitution on C3b binding by rhFH. An SPR experiment was performed as previously described to compare the affinity for immobilized C3b of LA-rhFH (a) with that of I62-rhFH (b). Graphs show plots of response vs concentration, with background-subtracted sensorgrams in inset. Figure adapted from (161). Performed by Dr Heather Kerr.

Table 5.4 shows that pre-incubation of LA-rhFH with PspCN in a two-fold molar excess produced minimal gain in affinity for C3b ($K_D = 0.70 \mu\text{M}$ *versus* $0.83 \mu\text{M}$ in one assay and $K_D = 0.60 \mu\text{M}$ *versus* $0.86 \mu\text{M}$ in another). No effect of the LA mutation on full-length rFH was seen in the DAA (assayed on SPR chip surface, Figure 5.18). These results, similar to those for D1119G-rhFH, suggest that (as expected) the N-terminal region of FH dominates its properties on the foreign surface of an SPR chip such that C-terminal mutations have minimal effects.

		K_D (μM)	$S.E$ (μM)	R_{max}	χ^2
Exp 1	I62-rhFH	1.2	0.20	175	5.2
	I62-rhFH + PspCN	0.5	0.07	266	31
	R53H-rhFH	1.8	0.30	217	6.5
	R53rhH + PspCN	1.0	0.10	310	13
	LA-rhFH	0.83	0.10	181	8.3
	LA-rhFH + PspCN	0.7	0.07	249	16
Exp 2	I62-rhFH	1.0	0.20	117	4.0
	I62-rhFH + PspCN	0.62	0.08	132	5.8
	R53H-rhFH	1.2	0.10	152	3.7
	R53rhH + PspCN	0.58	0.04	134	2.5
	LA-rhFH	0.86	0.11	146	6.6
	LA-rhFH + PspCN	0.60	0.07	151	8.0

Table 5.4 Summary of binding of factor H mutants with/without PspCN to C3b. SE: standard error. R : response units. χ^2 : chi². Table adapted from (161).

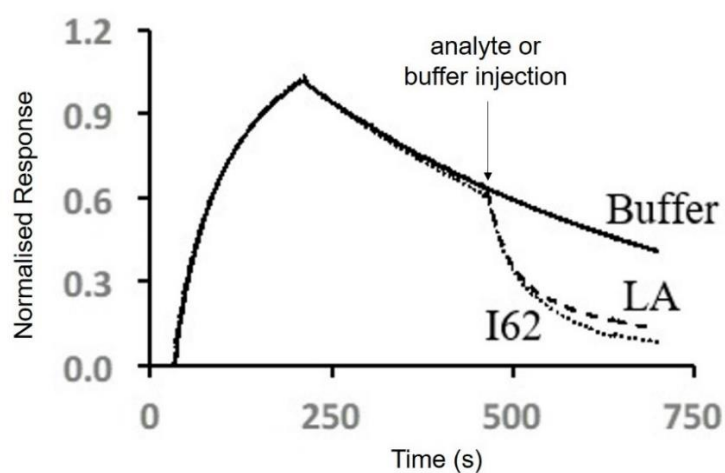


Figure 5.18 Effects of S1191L/V1197A substitutions in rhFH on its decay acceleration assay. An SPR-based assay of DAA was performed as shown in Figure 5.9 except that 40 nM LA-FH or 40 nM I62-FH were used. The data shown are representative of two experiments. Figure adapted from (161). Performed by Dr Heather Kerr.

When assayed on host-like E_S cells, the S1191L/V1197A substitutions had a smaller effect on DAA than on CA (Figure 5.19 a) and b)); the effect on C3bBb DAA was minor ($IC_{50} = 1.6$ nM for I62-rhFH *versus* $IC_{50} = 2.9$ nM for LA-rhFH) (Table 5.5), whereas in factor I cofactor assays, an IC_{50} of 63 nM for LA-rhFH was estimated, compared to an IC_{50} of 18 nM for I62-rhFH. This suggests that in the case of hFH acting to protect erythrocytes, sialic acid binding matters less for DAA than it does for CA. Interestingly, LA-rhFH was found to afford significantly less protection than wild-type (I62) rhFH to E_S in the haemolysis-protection assay (Figure 5.15 a). In further experiments on E_S , pre-incubation with (a 2:1 molar excess of) PspCN did not measurably increase the DAA of LA-rhFH. Conversely, it clearly enhanced the CA of LA-rhFH (Figure 5.19 c) and d)), although wild-type efficacy was not restored (Table 5.5). In haemolysis protection assays, the addition of PspCN was able to partially rescue LA-rhFH (Figure 5.15 a), as it had previously been found to do with the C-terminal mutant D1119G-rhFH (Figure 5.6 b). Moreover, PspCN also boosted the ability of LA-rhFH to protect the “foreign” surface of E_R (Figure 5.15 b).

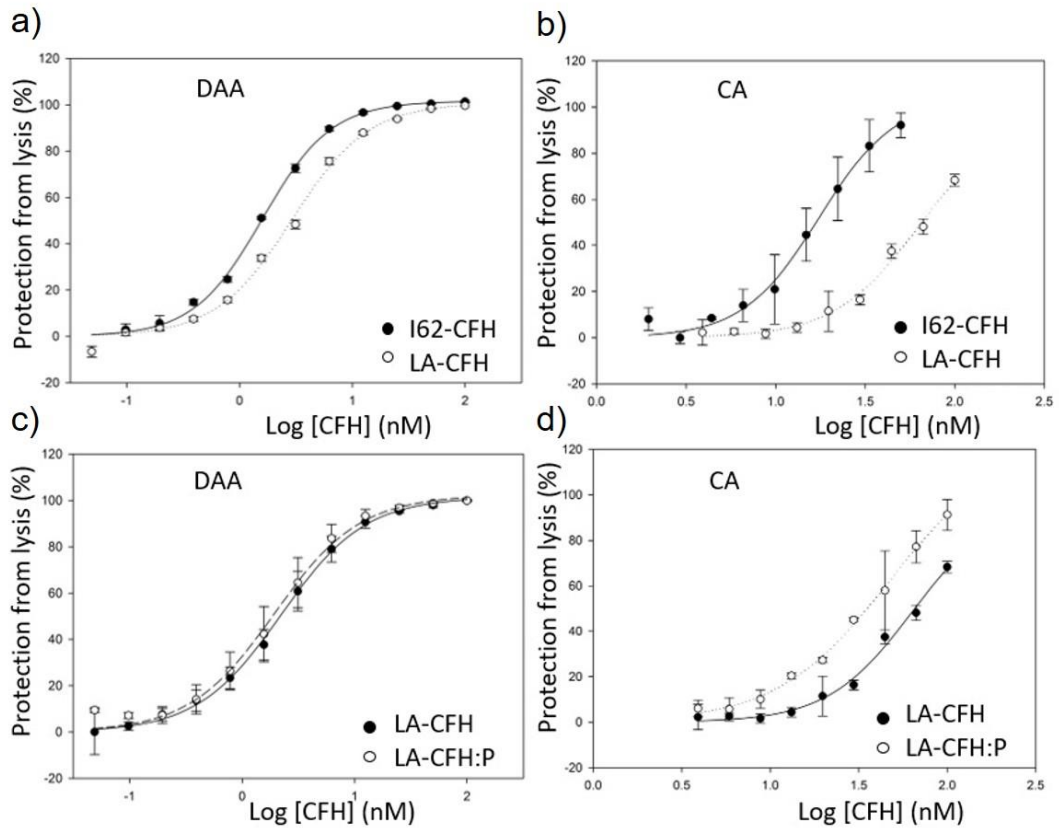


Figure 5.19 Effects of S1191L/V1197A substitutions in rhFH on erythrocyte-based assays. a) an E_S -based assay was carried out similar to that shown in Figure 5.16 a). b) an E_S -based assay was carried out similar to that shown in Figure 5.16 b). c) In an E_S -surface assay PspCN (2:1 to LA-rhFH) did not restore the DAA of LA-rhFH. d) In an E_S -surface assay, PspCN (2:1 to LA-rhFH) significantly enhanced the CA of LA-rhFH. Mean and S.D. (error bars) of triplicate measurements are plotted, and sigmoidal curves fitted using SigmaPlot. Each graph is representative of two experiments by Dr Edwin Wong. Figure adapted from (161).

Experiment	DAA IC ₅₀ (nM) ^a	Effect of the variation (or of PspCN) ^b	CA IC ₅₀ (nM) ^a	Effect of the variation (or of PspCN) ^b
I62-rhFH <i>versus</i> V62-rhFH	1.35 ± 0.10	0.94 ± 0.11	8.00 ± 0.33	1.19 ± 0.12
	1.27 ± 0.05		9.50 ± 0.57	
I62-rhFH <i>versus</i> I62-rhFH + PspCN	1.77 ± 0.05	(0.86 ± 0.05)	8.00 ± 0.33	(0.48 ± 0.03)
	1.52 ± 0.05		3.82 ± 0.06	
I62-rhFH <i>versus</i> R53H-rhFH	1.41 ± 0.44	4.72 ± 0.32	17.5 ± 0.8	2.44 ± 0.26
	6.65 ± 0.25		42.7 ± 2.5	
R53H-rhFH <i>versus</i> R53H- rhFH + PspCN	6.68 ± 0.6	(0.91 ± 0.15)	42.7 ± 2.5	(0.40 ± 0.04)
	6.06 ± 0.05		17.2 ± 0.6	
I62-rhFH <i>versus</i> D1119G-rhFH	1.33 ± 0.09	8.06 ± 1.31	12.0 ± 0.8	> 40
	10.7 ± 1.05		> 500	
D1119G-rhFH <i>versus</i> D1119G- rhFH + PspCN	9.21 ± 0.54	(1.24 ± 0.15)	> 500	
	11.5 ± 0.7		> 500	
I62-rhFH <i>versus</i> LA-rhFH	1.59 ± 0.06	1.83 ± 0.14	17.5 ± 2.6	3.63 ± 1.35
	2.92 ± 0.12		63.4 ± 14	
LA-rhFH <i>versus</i> LA-rhFH + PspCN	2.23 ± 0.16	(0.87 ± 0.14)	63.4 ± 14	(0.75 ± 0.38)
	1.94 ± 0.17		47.5 ± 13	

Table 5.5 Decay accelerating and co-factor activity on sheep erythrocytes.

^a Shown are the S.E. values for the fits of the data from each experiment. ^b The ratio of either IC₅₀ for the variant/IC₅₀ for I62-rhFH or (in parentheses) IC₅₀ for FH with PspCN/IC₅₀ for FH without PspCN. Figure adapted from (161).

5.2.5 Discussion

Nearly 100 variations in human *CFH*, the gene for FH, potentially predispose to aHUS or other diseases (88, 210). Most encode versions of FH that differ by a single amino acid residue, with only a fraction of the putative disease-linked versions of FH having been functionally characterised in any detail (88).

5.2.5.1 Factor H I62 and V62 have similar biological actions

A previously reported comparison between plasma-derived samples of V62-hFH and I62-hFH provided a backdrop to the study described here (163). Tortajada et al found the plasma-derived I62-hFH bound more efficiently to C3b and had enhanced co-factor activity in the FI-mediated cleavage of fluid phase and surface-bound C3b.

The recombinant versions of the two proteins used in the current research differ in sequence at position 936 (*i.e.* V62,E936-rhFH was compared with I62,D936-rhFH). The E936D SNP is not unique to at-risk or protective haplotypes (24, 148) nor located near the known binding sites for C3b, C3d or carbohydrates. The presence of Asp at position 936 protects against susceptibility to meningococcal disease (152) but is unlikely to impact our assays.

Recombinant V62-hFH and I62-hFH were found to be functionally equivalent to plasma-derived hFH in assays of C3b binding, decay acceleration and factor I CA. However, V62-rhFH and I62-rhFH bound much better than plasma-derived hFH to immobilized C3d (Figure 5.8 c). The most likely explanation for the difference in C3d binding between recombinant hFH and native hFH is the lack of N-glycans in the recombinant proteins. Native FH circulating in human plasma displays eight disialylated diantennary N-glycans (20, 24), some of which may contribute to the hidden nature of the TED/C3d-binding site in hFH CCPs 19 and 20 (See Section 5.1.3).

I62-rhFH bound marginally better than V62-rhFH to C3b in SPR-based comparison using identically prepared samples (Table 5.3) but more replicates would be required to establish significance. In further SPR experiments, pre-

incubation with PspCN enhanced C3b binding and the DAA of I62-rhFH and V62-rhFH to nearly identical extents. A slightly tighter complex with C3b formed by I62-hFH was reported in a previous SPR-based study conducted on plasma-derived hFH in “inverse mode” (*i.e.* C3b was flowed over immobilized FH)(163) (K_D values: I62-FH, 1.04 μM ; V62-FH, 1.33 μM). Comparisons of I62-hFH and V62-hFH are important because I62 is encoded by a SNP that is unique to a protective haplotype for AMD and C3 Glomerulopathy (148). A ‘complotype’ hypothesis (219) predicts that a coincidence of minor functional effects in several complement proteins could be additive in a patient. The I62-rhFH *versus* V62-rhFH comparison reported herein also agrees with the approximate parity of C3b binding between recombinant I62 and V62 variants of the truncated fragment rhFH CCPs 1-4 (203), despite rhFH CCPs 1-4 binding to C3b ($K_D = 11\text{-}14\ \mu\text{M}$) much less tightly than full-length hFH.

The SPR-derived K_D values reported in the literature for binding of FH to C3b vary severalfold (16, 23, 208), and the 0.2-0.3 μM K_D values obtained in the current three-way comparison are at the tighter end of this range. In other experiments (see Table 5.4), however, K_D values in the 1.0-1.3 μM range were obtained for the same samples. Such variations between SPR-derived K_D values were observed in this laboratory on numerous occasions. They probably reflect batch-to-batch variations of C3b or the sensor chip surface or both. Therefore, only measurements made on the same chip and in the same experiment can be meaningfully compared.

5.2.5.2 A CCP 1 mutation R53H has similar effects on self-surfaces and foreign surfaces

R53H is a mutation in CCP 1 of hFH. The consequences of this substitution in full-length rhFH were compared to those previously reported in the context of the truncated fragment rhFH CCPs 1-4 (122, 203). For R53H-rhFH versus I62-rhFH, the current study revealed: near-normal C3b-binding affinity (Figure 5.12); severalfold diminished complement regulatory functions on E_s (Figure 5.14); and negatively impacted C3bBb DAA in an SPR-based assay (Figure 5.13). These results closely parallel those obtained for R53H-rhFH CCPs 1-4.

These functional deficiencies are consistent with R53H causing aHUS (220), although it should be noted that most patients with this mutation are heterozygous. Moreover, 250 nM R53H-rhFH had wild type-like protective activity in the FH-reconstituted *E_s* haemolysis-protection assay (Figure 5.15 a). It could be that lack of control by R53H-rhFH leads to fluid-phase C3 depletion and a resultant, indirect, protection of *E_s* cells. To circumvent this potential anomaly we included along with R53H-rhFH equimolar amounts of I62-rhFH or LA-rhFH. I62-rhFH regulates complement both in fluid phase and on surfaces; LA-rhFH regulates complement in fluid-phase only. The I62:R53H combination, crudely emulating the situation in the plasma of a heterozygous patient, was also protective, probably because adequate I62-rhFH was present to achieve on-surface regulation as well as fluid-phase regulation. 1:1 LA:R53H-rhFH was not protective, demonstrating that R53H-rhFH could not protect *E_s* from haemolysis if C3 survives in the fluid-phase.

5.2.5.3 Characterisation of C-terminal C3b/d and sialic acid-binding site mutations reveal role of co-factor activity in self-surface complement regulation

Whereas easily prepared N-terminal truncation fragments of hFH, such as CCPs 1-4, serve as a good model for full-length hFH, the equivalent claim cannot be made for C-terminal fragments. More than 30 substitutions in CCPs 19-20 have been linked to aHUS (88). Cross-linking and other evidence (160, 221) suggests that the C-terminal CCPs of hFH modulate the activities carried out by the N-terminal modules of the same molecule. Therefore the use of full-length hFH is essential to fully characterise functional consequences of mutations in CCPs 19 and 20. Asp-1119 in CCP 19 is a key residue in the C-terminal C3d and C3b-binding site of hFH (16, 31). In the hFH 19-20 context, an aHUS-linked mutation, D1119G (220), severely reduced binding to C3b and C3d (16, 99). Moreover, D1119G-rhFH 19-20 was 40-fold less able than native sequence hFH 19-20 to compete with FH for binding to C3b-coated *E_s* (99). It was previously shown in this laboratory that full-length D1119G-rhFH, despite having wild type-like C3b-binding affinity and DAA activity on a foreign SPR chip surface, did not protect *E_s* from haemolysis (Figure 5.6 b). This has now

been taken further by showing that D1119G-rhFH diminishes 10-fold the ability of FH to accelerate decay of C3bBb assembled on E_S . The effect of D1119G-rhFH on the CA of FH, in the E_S context, is still greater.

This theme was further explored through studies of the aHUS-linked double mutation in CCP 20 of FH corresponding to a gene-conversion with CFHR-1 (*i.e.* S1191L/V1197A) (51). The crystal structure of S1191L-rhFH 19-20 bound to C3d is barely distinguishable from that of the native-sequence rhFH 19-20 complex (217). Conversely, LA-rhFH 19-20 failed to bind C3b-decorated E_S (99). Subsequently, Ser-1191 and Val-1197 were shown to contribute to the primary sialic acid-binding site in the hFH molecule (209). In current work it was found that LA-rhFH was significantly poorer than wild-type rhFH at protecting E_S from haemolysis by FH-depleted normal human serum (Figure 5.15 a). A similar result (Figure 5.15 c) was obtained using a 1:1 mixture of LA-rhFH and R53H-rhFH and yet in SPR experiments, the S1191L/V1197A substitutions had little impact on binding of rhFH to C3b. Moreover these mutations had only a small effect on C3bBb-DAA whether C3bBb was assembled on an SPR chip surface or on the surrogate host surface of E_S (Figure 5.19 a). Conversely, LA-rhFH was significantly deficient in CA on E_S cells. This distinction between DAA and CA, also seen in D1119G-rhFH, is further discussed below.

PspCN binds plasma hFH tightly, enhancing its ability to bind C3b and strongly increasing its affinity for C3d. The C3b/C3d-binding and self-recognition site in CCPs 19-20 of hFH becomes exposed upon binding PspCN, possibly emulating what happens when hFH binds to a self-surface. Herein, PspCN enhanced the affinities for C3b on an SPR-chip, of I62-rhFH, R53H-rhFH, and, to a lesser degree, LA-rhFH (Table 5.4). PspCN also increased the DAA of I62-rhFH and V62-rhFH on an SPR-chip (Figure 5.9). In contrast, PspCN did not increase the DAA of I62-rhFH on C3bBb-decorated E_S , and there was a similar lack of responsiveness to PspCN from R53H-rhFH, D1119G-rhFH and LA-rhFH in the E_S context (Table 5.5). The discrepancy between the two assays likely reflects the different surfaces employed: sialic acid-bearing and

self-like in the case of E_S , and “foreign” in the case of SPR. In contrast with its effects on E_S -surface DAA, PspCN significantly enhanced the CA of I62-rhFH on C3b-loaded E_S (Figure 5.11 d). It likewise had a positive effect, in this context, on the CA of R53H-rhFH (Figure 5.14 d), and it even partially rescued the CA of LA-rhFH (Figure 5.19 d). This difference between the responsiveness to PspCN of DAA and CA on E_S echoes differences between the impacts, on DAA (small) and CA (larger) of the C-terminal mutations S1191L/V1197A and D1119G.

The critical residues for DAA and CA reside in hFH CCPs 1-4. While the two activities regulate C3b amplification in a concerted manner, each involves a distinct sequence of events. To accelerate C3bBb decay, hFH probably binds simultaneously to both the surface-attached C3b and Bb (222) components of C3bBb, whereupon it acts catalytically to dissociate C3bBb into C3b and Bb. Following release of Bb, surface-tethered C3b retains affinity for hFH because both primary binding sites on C3b, engaged by FH CCPs 1-4 and 19-20, remain intact, as do interactions between CCP 20 and sialic acids on the surface. The hFH molecule must subsequently dissociate from C3b to catalyse another C3bBb-decaying event. As a co-factor, hFH binds to surface-attached C3b and recruits factor I (223). Subsequent cleavage of C3b to iC3b destroys its binding site for hFH CCPs 1-4 (29). Importantly, the binding site on iC3b used by CCPs 19 and 20, along with the interaction between CCP 20 and sialic acid, is not affected. The presence of the product, iC3b or C3d, on a sialic acid-decorated surface thus creates the possibility of retaining or enriching hFH on that surface by binding to the hFH C-terminus. Thus retained, these hFH molecules could utilise their CCPs 1-4 to act as a cofactor on nearby C3 molecules. In summary, complete detachment and reattachment of hFH from the surface is likely between cycles of DAA, but in the case of CA, FH can cycle between iC3b(C3d)-bound, iC3b(C3d)-plus-C3b-bound, and C3b-bound forms, without leaving the surface.

The results obtained herein for disease-linked C-terminal mutants on E_S surfaces support this line of reasoning because they show that retention of

hFH on a surface via its CCPs 19-20 is more important for CA than DAA. The results with PspCN support this reasoning because they confirm that the PspCN-induced increase in access to the surface-engaging C-terminal modules has little effect on DAA but a positive effect on CA.

5.2.5.4 Therapeutic potential of PspCN as a complement activator

To summarise, our results show that acceleration of C3bBb decay on a host-like surface is not very responsive to the presence or the absence (as tested by mutagenesis), or the availability or unavailability (tested by the inclusion or not of PspCN) of the self-recognition site in CCPs 19-20. On the other hand, CA (for FI on C3b bound to E_S) is sensitive to the presence or absence (mutagenesis) of the self-recognition site and its availability as enhanced by PspCN.

Soluble PspCN has a protective effect on E_R in haemolysis assays (Figure 5.15). This was observed previously and is consistent with bacterially displayed PspC preventing C3b amplification on the microbial surface. PspCN also partially rescued functionally deficient LA-rhFH in an E_S haemolysis-protection assay. These are more complex assays (compared with the individual DAA and CA assays) and less straightforward to interpret. However, we can infer that PspCN can work as a protector of self-surfaces mainly by enhancing FH CA on these surfaces, suggesting new therapeutic strategies based on FH activators. Indeed, Pouw et al (224) have shown that antibody-mediated potentiation of FH increased protection of primary human endothelial cells from complement, whilst preserving the complement-mediated killing of bacteria. The use of an 'activated' FH as a therapy may be an effective approach to inhibit unwanted complement activation on human cells in a wide range of diseases whilst preserving the protective role of complement against pathogens.

6: Recombinant murine factor H in a model of C3 Glomerulopathy

6.1 Background

Mice completely deficient in FH ($CFH^{-/-}$) spontaneously develop secondary plasma C3 deficiency and show deposition of C3 fragments along the glomerular basement membrane (GBM), in a model of C3 glomerulopathy (C3G) (119). Administration of serum-purified murine (m)FH resulted in the removal of C3 deposits along the GBM with an increase in plasma C3 levels at 24-hours post-injection (138). Additional studies showed that administering plasma-purified human (h)FH to $CFH^{-/-}$ mice also resulted in normalization of plasma C3 levels and resolution of GBM C3 deposition (139). However, long-term treatment with hFH was not possible as the mice developed an immune-complex glomerulonephritis and renal failure after ten days of daily hFH administration.

Experiment One, described below, was designed to compare the effects on $CFH^{-/-}$ mice of administering recombinant (r) mFH produced in yeast (*P. pastoris*) versus mFH purified from serum, at up to 24 hours after injection. Experiment Two aimed to compare the outcomes of ten daily injections of rmFH versus the same regimen of recombinant hFH (*i.e.* rhFH) injections. Due to the time-consuming challenge of achieving production of adequate quantities of rmFH, a study of extended dosing of $CFH^{-/-}$ mice with rmFH (as per the initial project aims) was not performed.

Dr Talat Malik (Centre for Complement and Inflammation Research, Imperial College London) was responsible for handling mice, giving injections, taking blood samples and processing kidneys. Dr Malik performed statistical tests using GraphPad Prism 8.0. Assays were performed by Heather Kerr unless otherwise stated.

6.2 Removal of Lipopolysaccharide

Bacterial endotoxins found in the outer membrane of gram-negative bacteria are members of a class of phospholipids called lipopolysaccharides (LPS). The

release of LPS from bacteria takes place after death and lysis of the microbial cell. LPS constitute the main contaminant in commercially available biological products such as recombinant proteins, and can cause side-effects including fever, hypotension, respiratory distress syndromes, intravascular coagulation, tissue injury and death (225). As such, there are strict guidelines from the United States Food and Drug Administration (USFDA) regarding acceptable endotoxin levels in biopharmaceutical materials (226). For intravenous applications, these must be lower than five endotoxin units (EU) per kg of bodyweight ($1 \text{ EU} = 10^{-4} \mu\text{g/mL}$). This can be scaled for murine models (226). Assuming a mouse weighs 30 g and based on realistic doses of protein, an endotoxin limit of 0.3 EU/mL was calculated for recombinant FH samples intended for administration to mice.

The depletion of LPS from FH samples was performed by Dr Jeremy Palmer (Institute of Cellular and Genetic Medicine, Newcastle University). The LPS was removed using phase separation with Triton X-114 (164). The endotoxin-depleted protein solution was then applied to Biobeads (Calbiochem) to remove any residual detergent. Levels of LPS were estimated before and after the procedure by measuring pyrogen-induced secretion of interleukin 6 (IL-6) from Mono Mac 6 cells. This assay is equivalent, for LPS detection, to the widely recognised Limulus Amoebocyte Assay (LAL) (165).

Figure 6.1 shows the amount of LPS measured (average $\pm$ SD for three samples) in initial aliquots of rmFH, and after each of four successive rounds of depletion. The rmFH preparation contained $\sim 2.5 \mu\text{g/mL}$ of LPS (25000 EU/mL) initially, but only $\sim 0.21 \times 10^{-4} \mu\text{g/mL}$ (0.2 EU/mL) after the LPS removal process. In the previous, reported study in which serum-purified mFH was administered to *CFH*^{-/-} mice, the sample contained 0.4 $\mu\text{g/mL}$ (4000 EU/mL) (138).

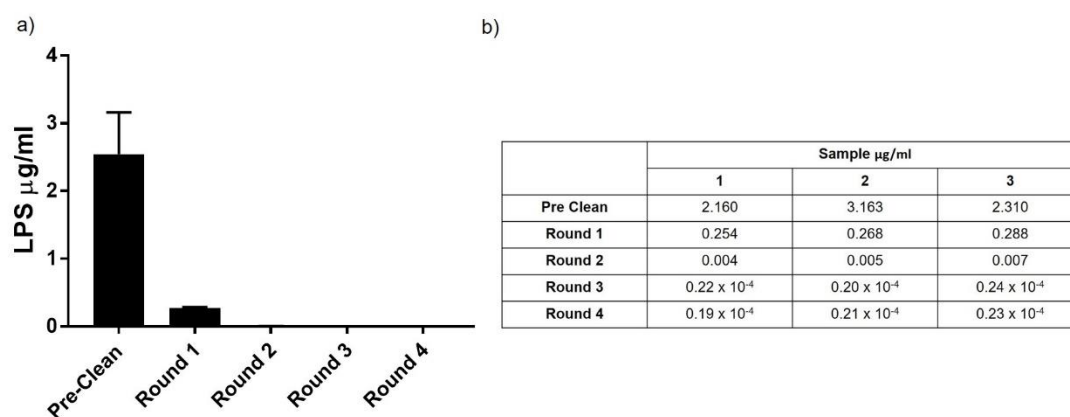


Figure 6.1 Depletion of LPS. a) Mean LPS levels, for three rmFH aliquots, before and after four sequential rounds of LPS depletion. Error bars denote the range of values. b) Individual values for LPS in each of the samples plotted in (a).

As a precaution, an additional LPS depletion process was conducted that resulted in undetectable levels of the endotoxin ($< 0.1 \times 10^{-4} \mu\text{g/ml}$).

Figure 6.2 shows that there does not appear to be significant degradation of the sample during the LPS-removal process and that levels of clipped protein (evident under reducing conditions) remain constant.

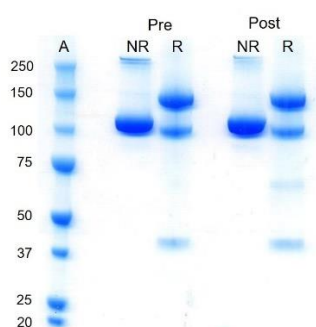


Figure 6.2 SDS PAGE of rmFH before and after LPS removal. A 5 µl aliquot of 1.5 mg/mL rmFH was loaded in each well and run under non-reducing (NR); or reducing (R) conditions. Lane A: protein MWt markers (kDa). There is evidence of clipped protein in lanes run under R conditions.

6.3 Experiment One: single intra-peritoneal dose of murine Factor H

Twelve *CFH*^{-/-} mice were available for this initial proof-of-concept study and this number determined the experimental design. Samples of rmFH were

administered to eight mice to determine whether plasma C3 levels could be restored and glomerular C3 staining reduced. Previous work had used either 1 mg of mFH (138) or 0.5 mg of hFH (139). Therefore four mice received 0.5 mg rmFH and four mice received 1.0 mg. In addition, two mice were given 0.5 mg of serum-derived mFH (this dose was limited by availability of the protein). The remaining two mice were administered vehicle only (*i.e.* 0.5 mL endotoxin-free PBS).

All injections were intra-peritoneal (*i.p.*) and the maximum volume injected was 0.5 mL. Mice were bled pre-injection, and at six hours and 24 hours post-injection. All mice remained alive prior to sacrifice at 24 hours post-injection and appeared healthy with no evidence of haematuria.

6.3.1 ELISA

6.3.1.1 Plasma Factor H

This assay was performed by Dr Hannah Lomax-Brown, Centre for Complement and Inflammation Research, Imperial College London.

Figure 6.3 shows plasma FH levels in *CFH*^{-/-} mice at pre-injection, six hours post-injection and 24 hours post-injection. A single 0.5-mL *i.p.* injection of either 0.5 mg rmFH, 1 mg rmFH, 0.5 mg mFH or PBS was administered. At six hours, all mice receiving FH unsurprisingly showed an increase in plasma FH levels, with the highest levels evident in mice receiving 1 mg of rmFH or 0.5 mg mFH.

By 24 hours, FH levels in the plasma of mice dosed with rmFH had decreased to approximately half the levels measured at six hours. Conversely, mice receiving serum-derived mFH had FH levels at 24 hours similar to those at six hours. Thus, rmFH appears to have been cleared more rapidly from the bloodstream compared to serum-derived mFH.

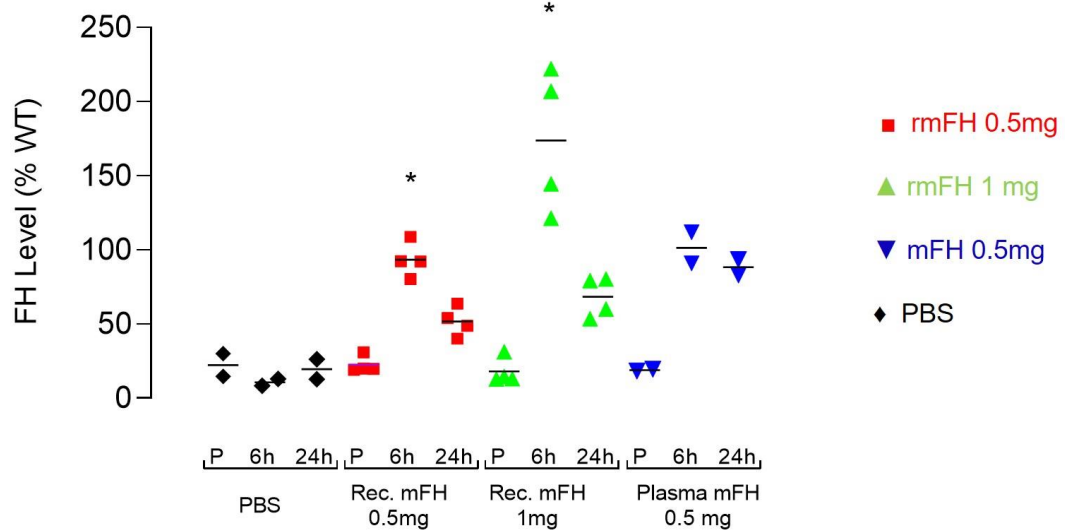


Figure 6.3 Plasma FH levels in $CFH^{-/-}$ mice after a single injection of rmFH, mFH or PBS. Mouse plasma FH levels as a percentage of wild-type (WT) FH levels after single *i.p.* injection of 0.5 mg rmFH, 1 mg rmFH, 0.5 mg mFH or PBS alone. P – pre; Rec. – recombinant, mFH – mouse factor H. Horizontal bars denote mean values. * $P=0.0034$ vs pre-injection value, Dunn's Multiple Comparisons.

6.3.1.2 Plasma C3

This assay was performed by Dr Talat Malik.

Figure 6.4 shows C3 levels in $CFH^{-/-}$ mice plasma at the same three time points as in Figure 6.3, *i.e.* pre-injection, six hours post-injection and 24 hours post-intraperitoneal injection of either 0.5 mg rmFH, 1 mg rmFH, 0.5 mg mFH or PBS (as above).

At six hours, all mice receiving murine FH showed increased levels of C3 in plasma, with the highest levels being seen in mice that had received serum-derived mFH (mean values: 180 $\mu\text{g/mL}$ C3 in mice receiving 0.5 mg rmFH, 241 $\mu\text{g/mL}$ in mice receiving 1 mg rmFH, and 298 $\mu\text{g/mL}$ in mice receiving mFH). Note that, by way of comparison, a mean C3 concentration of 228 $\mu\text{g/mL}$ ($\pm 37.5 \mu\text{g/mL}$) was measured in pooled plasma from non-dosed, WT mice using this ELISA.

At 24 hours, mice that had received rmFH showed decreased plasma levels of C3 compared to those measured at six hours. Mean values in mice receiving

0.5 mg and 1 mg rmFH were 87 $\mu\text{g/mL}$ and 120 $\mu\text{g/mL}$, respectively, compared to 365 $\mu\text{g/mL}$ in mFH-dosed mice.

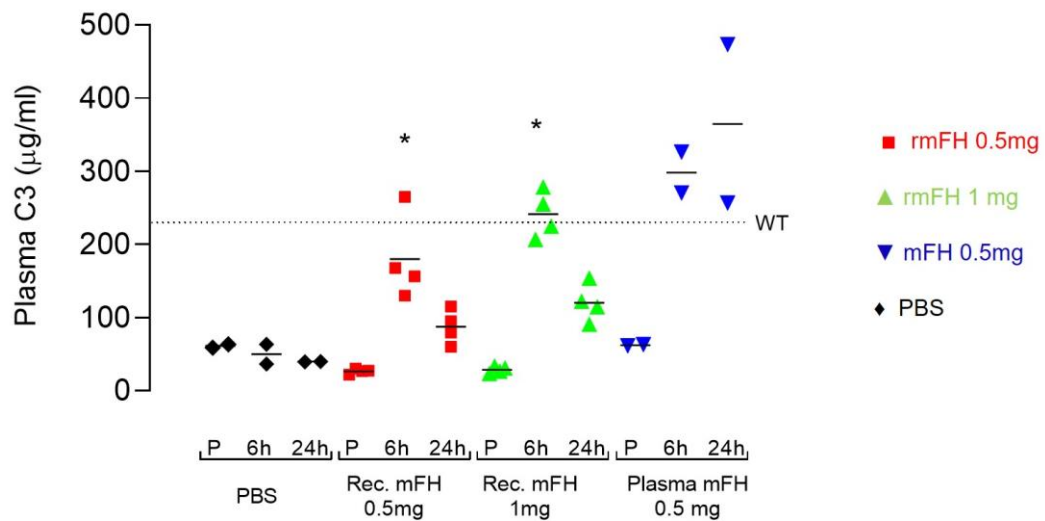


Figure 6.4 Plasma C3 levels in *CFH*^{-/-} mice after injection with rmFH, mFH or PBS. Mouse plasma C3 levels after single *i.p.* injection of 0.5 mg rmFH, 1 mg rmFH, 0.5 mg mFH or PBS alone. WT – wild-type, P – pre; Rec. – recombinant, mFH – mouse factor H. Horizontal bars denote mean values. WT – mean plasma C3 concentration, 228 $\mu\text{g/mL}$. * $P=0.0034$ vs pre-injection value, Dunn's Multiple Comparisons.

The reduction in plasma C3 levels at 24 hours in mice dosed with recombinant FH is consistent with the fall in plasma FH levels seen at 24 hours (see Fig. 6.3).

6.3.2 Western Blot for Plasma C3

This assay was performed by Dr Heather Kerr.

Complement activation involves cleavage of the α -chain of C3 to release C3a and form C3b. The intact α -chain of murine C3 runs on reducing SDS-PAGE at approximately 115 kDa, whilst the β -chain runs at 55-60 kDa (145, 227). Figure 6.5 shows a Western blot used to identify the chains of C3 (under reducing conditions) recovered from the plasma of wild-type (WT) or *CFH*^{-/-} mice that had received rmFH, mFH or PBS. C3 in the plasma of WT mouse had predominantly intact α - and β -chains. There was a minor presence of fragments that seem to have arisen due to repeated freeze/thaw of the sample

(personal communication, Dr Marieta Ruseva, Centre for Complement and Inflammation Research, Imperial College London).

The plasma of *CFH*^{-/-} mouse that had received PBS alone showed a lack of a defined band for C3 β -chain, no detectable band for intact α -chain, and a band that could correspond to fragments of α -chain. This is consistent with expectations since the lack of FH will allow continuous AP activation and depletion of C3. At six hours post injection, the presence of both intact C3 α -chain and β -chain was evident in all *CFH*^{-/-} mice that had received injections of FH. By 24 hours, in mice that had received rmFH, cleaved α -chain fragments are present while intact α -chain and β -chain are absent. This loss of fluid-phase AP regulation in mice receiving rmFH, after 24 hours, is consistent with the decrease in FH observed by ELISA in Figure 6.3, and the loss of C3 shown by ELISA in Figure 6.4. Conversely, *CFH*^{-/-} mice treated with serum-derived mFH continue to show evidence of AP regulation at 24 hours post-injection.

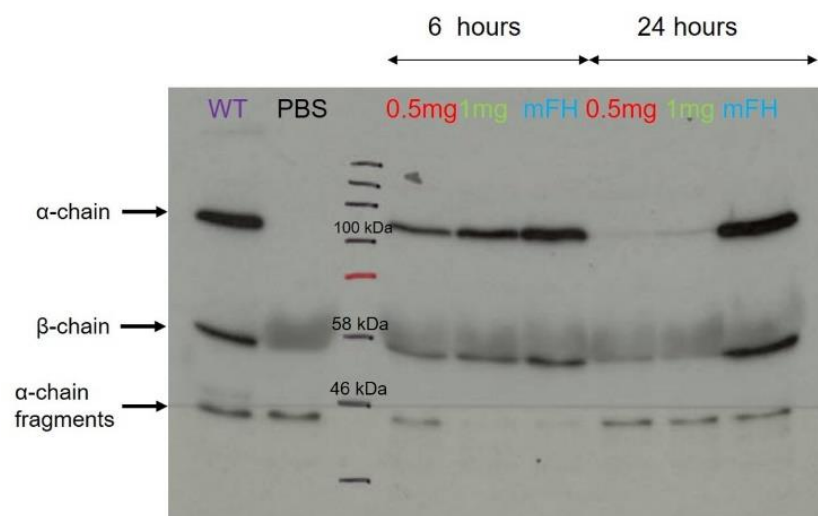


Figure 6.5 Western blot for C3 in plasma of *CFH*^{-/-} mice after doses of rmFH or mFH. Mice received at time zero a single *i.p.* injection of PBS (vehicle), 0.5 mg rmFH, 1 mg rmFH or 0.5 mg mFH (results for PBS are for blood taken at the 24-hour point only). WT: wild-type mouse (no treatment). Samples (1 μ l of mouse plasma per well) were run under reducing conditions. Primary antibody: goat anti-mouse C3 (MP Biomedicals); secondary antibody: anti-goat IgG HRP (Sigma).

6.3.3 Glomerular Immunostaining

6.3.3.1 C3

Glomerular immunostaining and quantification were performed by Dr Talat Malik.

Figure 6.6 shows representative images of glomerular C3 immunostaining in *CFH*^{-/-} mice, 24 hours after injection of rmFH, mFH or PBS. In *CFH*^{-/-} mice injected with PBS, linear capillary wall staining with an anti-C3 antibody is evident as previously described (119, 138, 139). In mice injected with rmFH, the pattern of capillary wall staining was markedly reduced compared to PBS-injected mice. In mice that had received mFH the pattern of capillary wall staining was also reduced, but in addition there was evidence of tubulo-interstitial staining (indicative of levels of circulating C3 being restored), as was also seen in healthy WT mice (138). These results are fully consistent with the higher circulating plasma FH and C3 levels observed after 24 hours in mice that had received mFH (Figures 6.3 and 6.4).

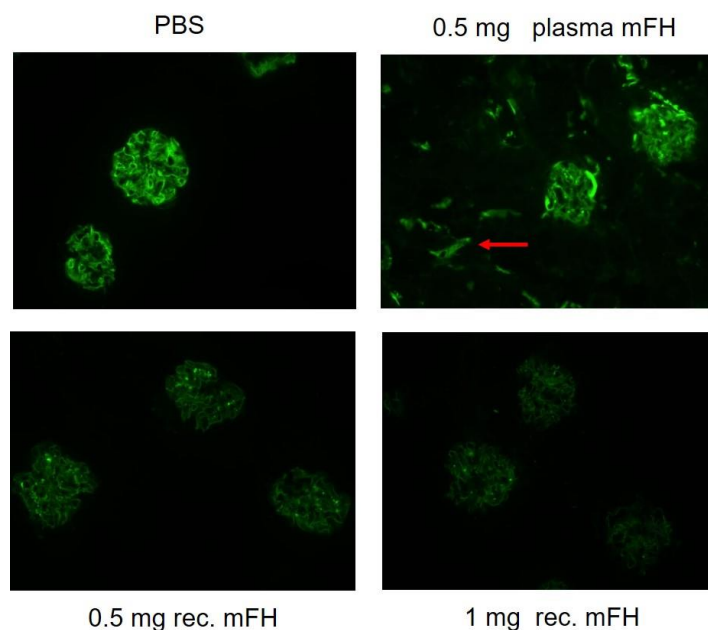


Figure 6.6 Representative images of glomerular C3 immunostaining in *CFH*^{-/-} mice 24 hours after injection with the stated amounts of rmFH or mFH, or PBS (vehicle). Antibody: goat anti-mouse C3 (MP Biomedicals). Arrow denotes tubulo-interstitial staining.

Figure 6.7 shows that immunostaining for C3 in glomeruli at 24 hours post-injection reduced to similar extents in *CFH*^{-/-} mice receiving rmFH and mFH, compared to mice receiving PBS alone (mean values: mice receiving 0.5 mg rmFH, 25 AFU (arbitrary fluorescent units); mice receiving 1.0 mg rmFH, 19 AFU; mice receiving 0.5 mg mFH 29 AFU; PBS-treated mice 80 AFU). This near equivalence in the reduction of C3 deposition was unexpected given the lack, at 24 hours, of both FH and C3 in plasma of mice treated with rmFH.

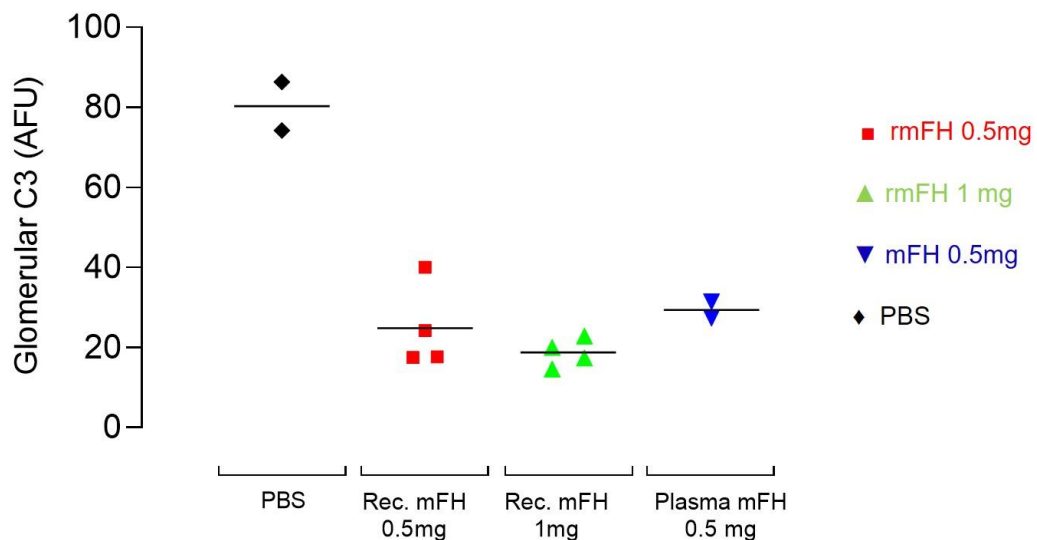


Figure 6.7 Results of glomerular C3 immunofluorescence measurements. Following injection of *CFH*^{-/-} mice with rmFH, mFH or PBS, glomeruli were immunostained, at the 24-hour time point, for C3. AFU: arbitrary fluorescent units. Horizontal bars denote mean values.

6.3.3.2 IgG

This assay was performed by Dr Heather Kerr.

Figure 6.8 shows representative images of glomerular IgG immunostaining, 24 hours after injection into mice of rmFH, mFH or PBS. IgG staining at the GBM would indicate formation of immune complexes, such as might arise from the presence of anti-FH antibodies. No evidence of IgG staining is seen.

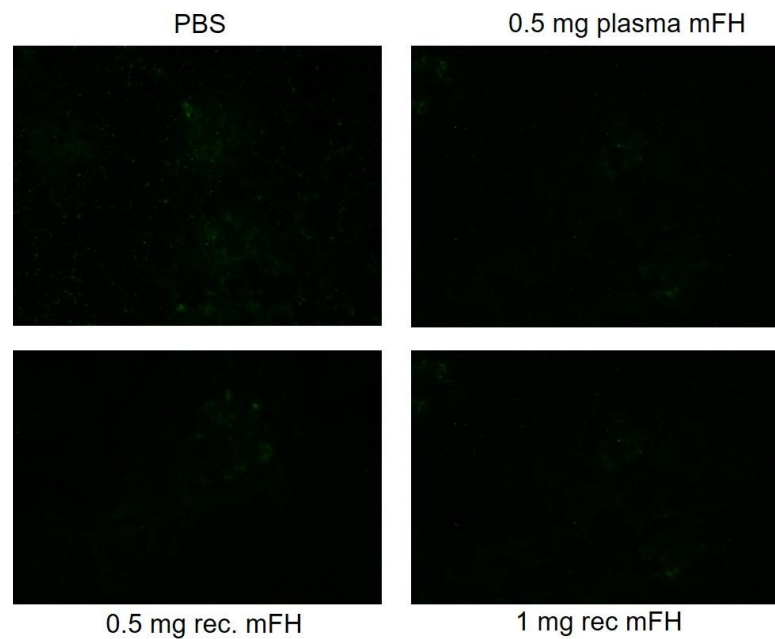


Figure 6.8 Representative images of glomerular IgG immunostaining in *CFH*^{-/-} mice 24 hours after injection of rmFH, mFH or PBS. Antibody: goat anti-mouse IgG (Sigma).

6.3.4 Summary of Experiment One

In sum, these studies show: i) high levels of mFH and intact C3 persist in plasma 24 hours after dosing *CFH*^{-/-} mice with native FH; ii) dosing with rmFH temporarily raised plasma mFH levels but rmFH was cleared faster than native mFH; iii) presumably due to faster clearance, injected rmFH controlled fluid-phase C3 consumption less effectively than did native mFH; iv) the protective effects of rmFH, in terms of preventing C3 fragment deposition along the GBM, persisted after serum levels of the administered recombinant protein had fallen to 50% of wild-type levels.

6.4 Experiment Two: daily intra-peritoneal dosing with recombinant Factor H

Twelve *CFH*^{-/-} mice were available for this experiment that (inspired by the rapid clearance of rmFH in Experiment One) was designed to study the cumulative outcome of regular injections of FH over a period of ten days. In Experiment One, treatment with 1 mg of rmFH achieved plasma C3 levels comparable to WT levels (Figure 6.3). Doses of 0.5 mg rmFH were used in the

daily-dose experiments to conserve sample and, potentially, to allow for a gradual build up of FH levels. It was decided to include some mice that had received recombinant human FH (rhFH) for comparison and as a control. Available stocks of rhFH limited daily doses to 0.4 mg rhFH and n (mice) = 2. Another four mice received 0.5 ml PBS (vehicle controls). A comparison with serum-derived mFH was not possible due to the lack of availability of protein.

Thus, for this experiment, five mice were injected daily for ten days with 0.5 mg recombinant murine FH (rmFH), two mice were injected daily for ten days with 0.4 mg recombinant IYD allotypic variant of human FH (I62-rhFH), and five mice were injected daily for ten days with 0.5 mg endotoxin-free PBS (Sigma). Injections were intra-peritoneal in a volume of 0.5 ml. Mice were bled pre-injection, at 24 hours, five days and 11 days (*i.e.* 24 hours after the final injection). A mouse (mouse 4) that received PBS only, died on day 3. A mouse that had rhFH was noted on day 10 to have evidence of macroscopic haematuria. Surviving mice were sacrificed on day 11.

6.4.1 ELISA

6.4.1.1 Plasma factor H

This assay was performed by Dr Hannah Lomax-Brown.

Figure 6.9 a) shows levels of FH (normalised to FH levels in the plasma of WT mice) in the plasma of $CFH^{-/-}$ mice pre-injection, and at 24 hours, five days and 11 days into a regimen of daily *i.p.* injections of rmFH (or PBS). Figure 6.9 b) shows levels of FH in the plasma of $CFH^{-/-}$ mice receiving rhFH at the same time points. Results for administration of rmFH and rhFH are shown on different graphs because FH was measured using anti-human FH antibodies, which bind much better to rhFH protein than to rmFH; hence optical density readings for hFH were too high to be plotted accurately in the initial ELISA.

At 24 hours, mice receiving 0.5 mg rmFH had plasma levels of FH comparable to those in mice receiving 0.5 mg rmFH in Experiment One (Experiment One, 52% of WT level; Experiment Two, 51% of WT level). Plasma FH levels were found to be reduced by day five (mean value 44% of WT) despite the daily

doses of FH. By day 11, despite animals receiving ten doses (5 mg in total) of FH, their plasma FH levels had dropped to 18 % (mean value) of plasma levels in WT mice. Therefore, despite an extended daily dosing regime, it seems that the rmFH was cleared from the bloodstream as quickly as it was added.

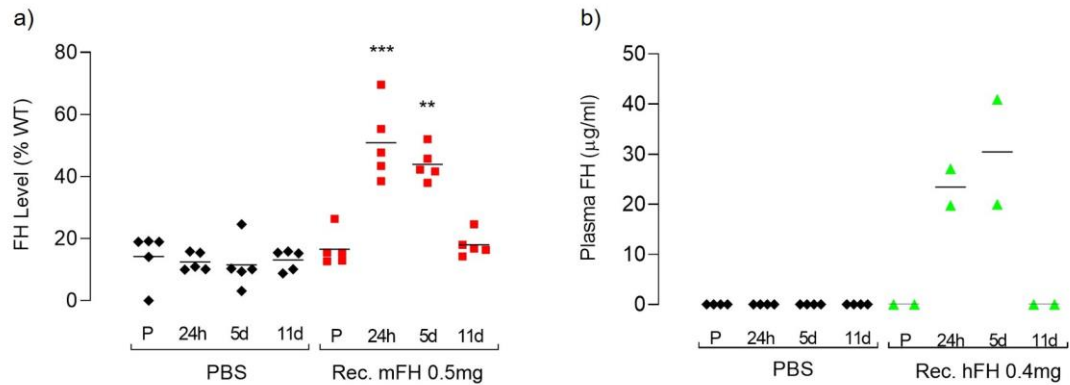


Figure 6.9 Plasma FH levels in $CFH^{-/-}$ mice following ten daily injections with rmFH, rhFH or PBS. a) Levels of FH in mouse plasma - plotted as a % of WT FH levels - after ten daily *i.p.* injections of 0.5 mg rmFH (or PBS alone). b) Levels of FH in mouse plasma following ten daily *i.p.* injections of 0.4 mg rhFH (or PBS alone). Horizontal bars denote mean values. ** $P=0.0255$, *** $P=0.0033$ vs pre-injection value, Dunn's Multiple Comparisons.

In Figure 6.9 b) it appears that rhFH levels are higher after five days of daily injections than at 24 hours (mean values of 30.45 µg/ml *versus* 23.43 µg/ml respectively), but with $n = 2$ only it is not possible to attach significance to these differences.

6.4.1.2 Plasma C3

This assay was performed by Dr Heather Kerr.

Figure 6.10 a) and b) show levels of C3 in the plasma of $CFH^{-/-}$ mice, pre-injection, and at 24 hours, five days and 11 days (*i.e.* 24 hours after the final injection) - after daily *i.p.* injections of 0.5 mg rmFH, 0.4 mg rhFH or PBS (vehicle).

At 24 hours, mean C3 levels in the plasma of mice in receipt of rmFH was 78 µg/ml. This compares to 93 µg/ml for mice receiving rhFH, while for vehicle controls it was 38 µg/ml. Thus in 0.5-mg rmFH-treated mice, C3 levels were

comparable to those (87 $\mu\text{g}/\text{mL}$, see Figure 6.4) observed at the same time point in Experiment One.

After five daily doses, mean plasma C3 levels were 43 $\mu\text{g}/\text{mL}$ for rmFH-treated mice, 65 $\mu\text{g}/\text{mL}$ for rhFH-treated mice, and 41 $\mu\text{g}/\text{mL}$ for mice that had received PBS. At the end of the treatment regime (11 days) mean plasma C3 levels dropped to 20 $\mu\text{g}/\text{mL}$ for rmFH-treated mice, 48 $\mu\text{g}/\text{mL}$ for rhFH-treated mice, and 19 $\mu\text{g}/\text{mL}$ for mice receiving only PBS.

Although only two mice received rhFH, it is notable that at all time points mean plasma C3 levels were higher than those of mice dosed with rmFH. Mean plasma C3 levels for control mice that received vehicle also appear to rise and then fall during the dosing regimen (25 $\mu\text{g}/\text{mL}$ pre-dose, 38 $\mu\text{g}/\text{mL}$ after 24 hours, 41 $\mu\text{g}/\text{mL}$ after five days, 19 $\mu\text{g}/\text{mL}$ after 11 days). A similar phenomenon was observed previously (139, 228).

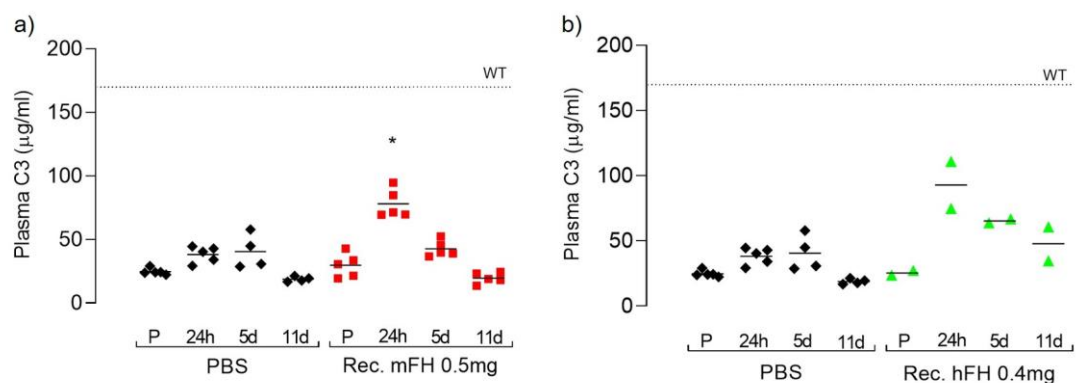


Figure 6.10 Plasma C3 levels in $CFH^{-/-}$ mice receiving ten daily injections of rmFH, rhFH or PBS. Mice were *i.p.* injected daily for ten days with (a) 0.5 mg rmFH, or PBS alone or (b) 0.4 mg rhFH or PBS alone. Horizontal bars denote mean values. WT – mean plasma C3 concentration, 165 $\mu\text{g}/\text{mL}$. *P=0.0192 vs pre-injection value, Dunn's Multiple Comparisons.

A previous study in which $CFH^{-/-}$ mice received daily doses of purified human FH for ten days, reported that, at five days, plasma C3 reached WT levels (139) (~200 $\mu\text{g}/\text{mL}$). Conversely, in the current study, plasma C3 levels at the day five time point, remained lower than WT levels for rhFH-treated mice and were almost baseline for mice receiving rmFH (mean of 30 $\mu\text{g}/\text{mL}$ pre-dosing,

versus 43 $\mu\text{g/ml}$ at five days). Such low plasma C3 levels are below what was expected given the elevated FH levels at this point (Figure 6.9).

6.4.1.3 Anti-factor H antibodies

This assay was performed by Dr Heather Kerr.

Figure 6.11 shows the outcome of attempts to detect anti-FH antibodies in *CFH*^{-/-} mice plasma, at various time points, after injection of rmFH, rhFH or PBS. There is no evidence of anti-FH antibodies at any time point other than 11 days. In the two mice that received rhFH, anti-FH antibody levels appear higher than in the mice that received rmFH. In the previous study in which human serum-derived FH was given to *CFH*^{-/-} mice, anti-FH antibodies were also seen after ten daily doses (139). It had been postulated that administration of murine FH may result in less anti-FH antibody formation compared to administration of human FH, because of the presence of Factor H-related proteins in mice (39, 84, 86) that likely induce partial immune tolerance to exogenous murine FH administration.

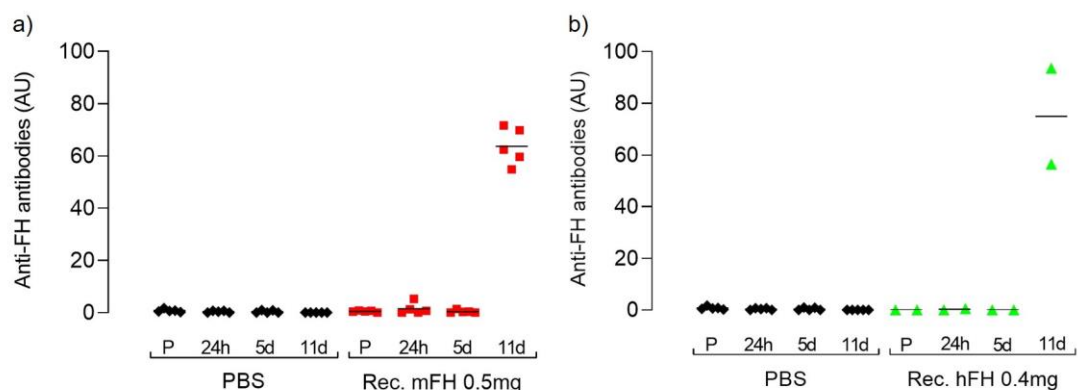


Figure 6.11 Plasma anti-FH antibody levels in *CFH*^{-/-} mice after injection with rmFH, rhFH or PBS. a) Mouse plasma anti-FH antibody levels after ten daily *i.p.* injections of 0.5 mg rmFH or PBS alone. b) Mouse plasma anti-FH antibody levels after ten daily intra-peritoneal injections of 0.4 mg rhFH or PBS alone. Horizontal bars denote mean values.

6.4.2 Western Blot for Plasma C3

This assay was performed by Dr Heather Kerr.

Figure 6.12 shows a Western blot highlighting components of C3 (under reducing conditions) in two *CFH*^{-/-} mice at day 1, day 5 and day 11 after daily injections of rmFH. As noted above, the plasma of wild-type (WT) mouse – included for comparison in Figure 6.12 – contains high levels of intact α -chain and β -chain; while some α -chain fragments are detected in the plasma of the WT mouse, these likely arise from repeated freeze/thaw of the sample.

As reported above, the *CFH*^{-/-} mouse that received vehicle had no detectable intact β -chain or α -chain in their plasma but significant levels of α -chain fragments. According to Figure 6.5 (Experiment One), the α -chain of C3 had re-appeared at six hours after administration of rmFH, but faded by 24 hours accompanied by reappearance of α -chain fragments. In Experiment Two, intact α -chain is present at 24 hours along with some α -chain fragments, but intact α -chain was not detectable at the final, 11-day, time point. This is consistent with the results shown in Figure 6.10, where plasma C3 levels are at baseline by the end of the dosing regime.

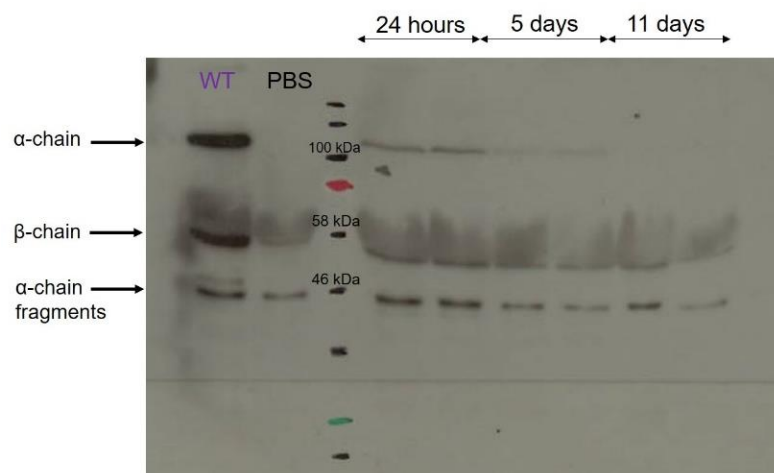


Figure 6.12 Western blot of plasma C3 levels in two *CFH*^{-/-} mice receiving injections of rmFH. Mouse plasma C3 after single *i.p.* injections of rmFH at 24 hours, and after daily injections at five days and 11 days (24 hours after the last injection). WT: wild-type mouse. PBS: *CFH*^{-/-} mouse that had received PBS only, for ten days, at 24 hours post-final injection. Each well was loaded with 1 μ l mouse plasma and run under reducing conditions. Primary antibody: goat anti-mouse C3 (MP Biomedicals); secondary antibody: anti-goat IgG HRP (Sigma).

6.4.3 Glomerular Immunostaining

6.4.3.1 C3

This assay was performed by Dr Heather Kerr.

Figure 6.13 shows representative images of glomerular C3 immunostaining at day 11, *i.e.* 24 hours after the final injection of rmFH, rhFH or PBS. In control *CFH*^{-/-} mice injected with vehicle, linear capillary wall staining with an anti-C3 antibody is evident as described previously (119, 138, 139). Importantly, in mice injected with either rmFH or rhFH, the pattern of capillary wall staining is markedly reduced compared to control (vehicle only) mice. This contrasts with a previously published ten-day study with human serum-derived FH (139), where despite a marked reduction in C3 staining after five days, strong C3 staining had returned by the end of the dosing regime.

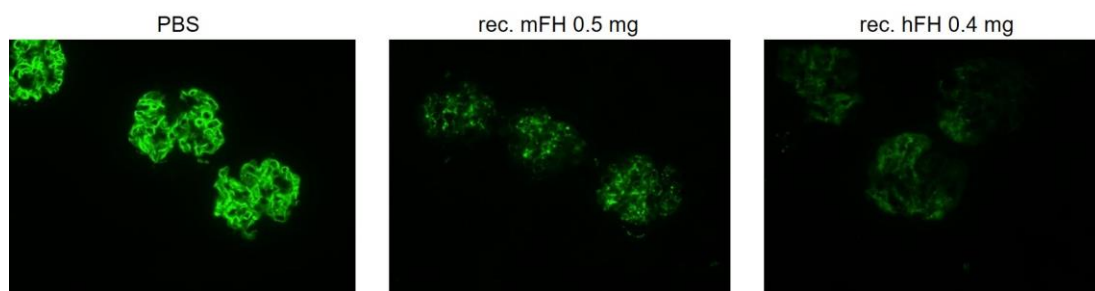


Figure 6.13 Representative images of glomerular C3 immunostaining in *CFH*^{-/-} mice after ten daily injections of rmFH, rhFH or PBS. Antibody: goat anti-mouse C3 (MP Biomedicals).

Figure 6.14 confirms that glomerular C3 immunostaining after ten daily injections remains reduced in *CFH*^{-/-} mice receiving either rmFH or rhFH compared to mice receiving PBS (mean values: rmFH, 42 AFU; rhFH, 50 AFU; PBS 127 AFU). This is despite reductions in plasma FH/C3 levels by this time-point.

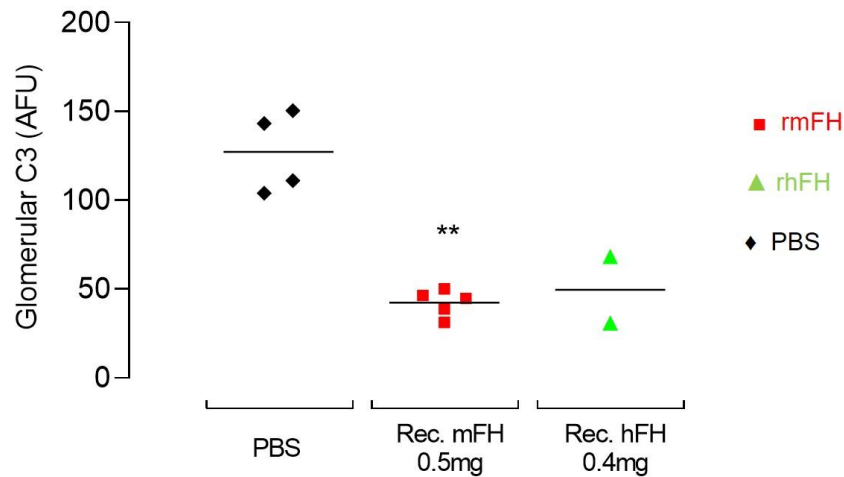


Figure 6.14 Glomerular C3 fluorescent intensity in *CFH*^{-/-} mice after ten daily injections with rmFH, rhFH or PBS. AFU: arbitrary fluorescent units. **P=0.0159 vs PBS, Mann-Whitney Test. Horizontal bars denote mean values.

6.4.3.2 C3d

This assay was performed by Dr Heather Kerr.

Figure 6.15 shows representative images of glomerular C3d immunostaining at day 11. In control mice injected with vehicle, there is evidence of glomerular reactivity with an anti-mouse C3d antibody (this antibody does not recognise C3, iC3b, C3c or C3b). Reduced staining can be seen in mice that had received daily rmFH or rhFH. This is in concordance with a previously reported reduction in glomerular C3d staining observed in studies using human serum-derived FH (139). However, the Fakhouri *et al.* study also showed tubulo-interstitial staining for C3d (which had not been seen at 5 days). No evidence of tubulo-interstitial staining is seen in Figure 6.15.

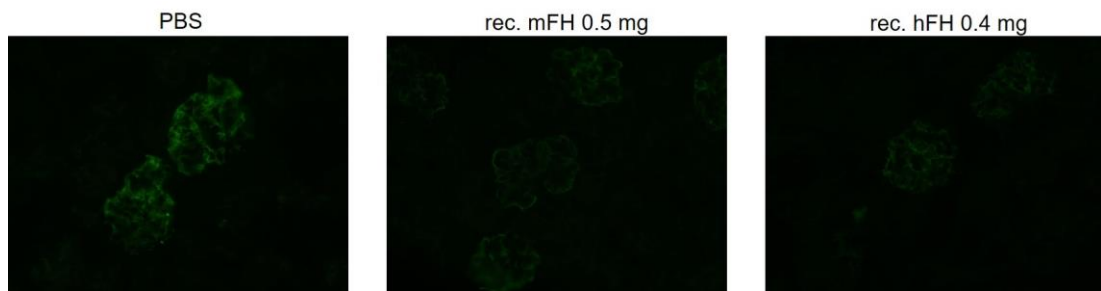


Figure 6.15 Representative images of glomerular C3d immunostaining in *CFH*^{-/-} mice after ten daily injections of rmFH, rhFH or PBS. Antibody: biotinylated goat anti-mouse C3d (R + D Systems).

Figure 6.16 shows that glomerular C3d immunostaining 24 hours after the last of ten daily injections is reduced to a similar extent, both in *CFH*^{-/-} mice receiving rmFH (326 AFU) and those receiving rhFH (282 AFU), in comparison to vehicle-only controls (450 AFU). This is despite the reduction of both plasma FH and plasma C3 levels by this time-point.

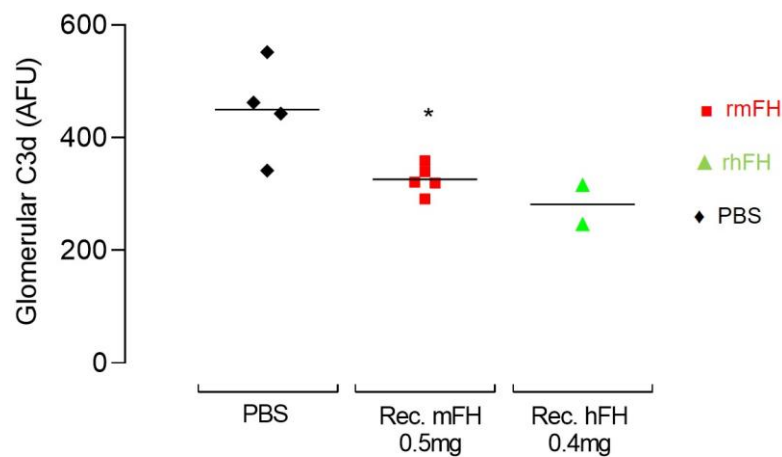


Figure 6.16 Glomerular C3d fluorescent intensity in *CFH*^{-/-} mice after ten daily injections with rmFH, rhFH or PBS. AFU: arbitrary fluorescent units. Horizontal bars denote mean values. *P = 0.0317 vs PBS, Mann Whitney test.

6.4.3.3 IgG

This assay was performed by Dr Heather Kerr.

Figure 6.17 shows representative images of glomerular IgG immunostaining at day 11 of the daily dose regimen. No evidence of IgG staining is seen in vehicle-only control mice. Florid IgG staining is seen in mice that received rhFH – this also occurred in a previous study with human serum-derived FH (139). There was a varied response in mice dosed with rmFH; some animals exhibiting milder evidence of IgG staining and others a more florid picture (although not as severe as with rhFH). The deposits in Figure 6.17 d) in particular appear granular, consistent with immune complex deposition.

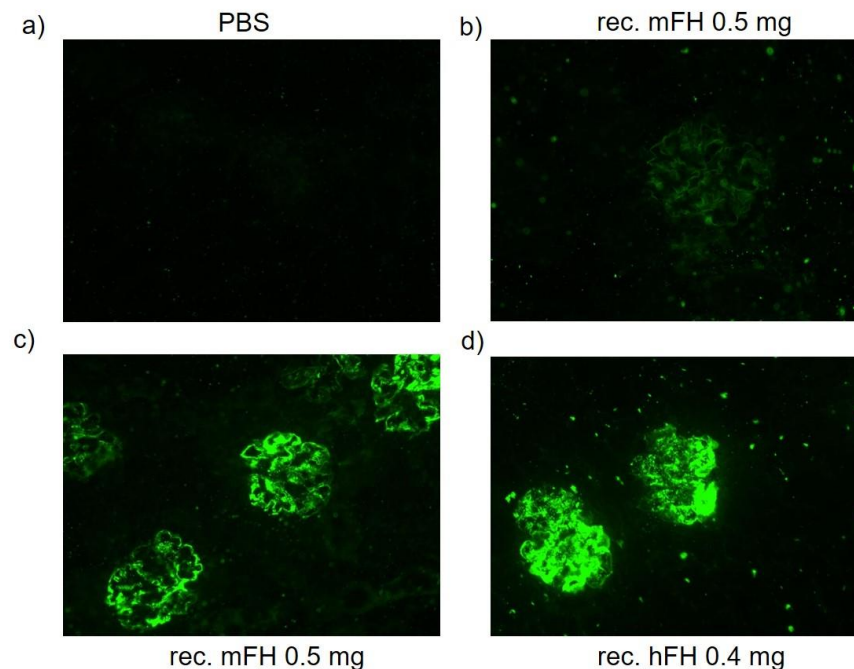


Figure 6.17 Representative images of glomerular IgG immunostaining in *CFH*^{-/-} mice 24 hours after the last of ten daily injections of rmFH, rhFH or PBS. a) PBS b) rmFH 0.5 mg c) rmFH 0.5 mg d) rhFH 0.4 mg. Antibody: goat anti-mouse IgG (Sigma).

Figure 6.18 shows that glomerular IgG fluorescent intensity appears most pronounced in mice that have received rhFH (mean values: rmFH 515 AFU; rhFH 1776 AFU; PBS 95 AFU). This is in agreement with Figure 6.11, in which the presence of anti-FH antibodies appears highest in those mice dosed with

rhFH (although $n = 2$). In the previously reported study with human serum-derived FH (139), strong glomerular FH staining was seen after ten days in a pattern equivalent to that seen for IgG, and suggested the deposition of mouse IgG-hFH immune complexes in the kidney. FH staining of the glomeruli was not performed in the current study, but it is likely that IgG staining in Figure 6.17 also represents mouse IgG-rmFH or mouse IgG-rhFH immune complex deposition in the kidney.

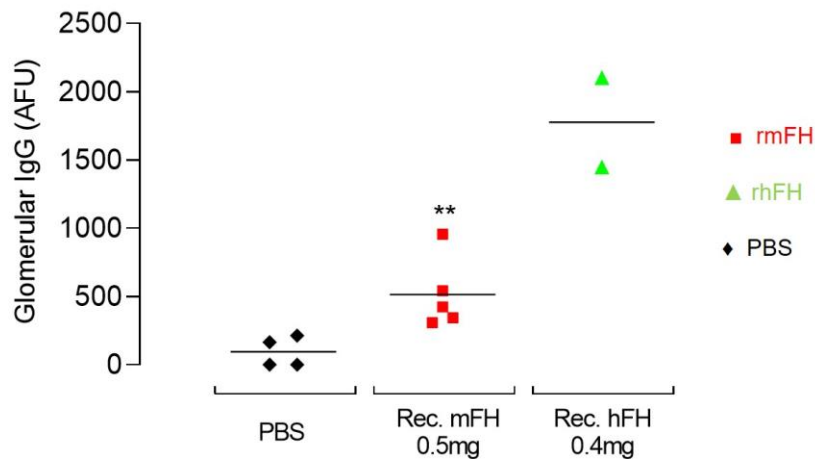


Figure 6.18 Glomerular IgG fluorescent intensity in $CFH^{-/-}$ mice 24 hours after the last of ten daily injections with rmFH, rhFH or PBS. AFU: arbitrary fluorescent units. ** $P=0.0159$ vs PBS, Mann-Whitney Test. Horizontal bars denote mean values.

6.4.4 Summary of Experiment Two

After five days of daily dosing, $CFH^{-/-}$ mice receiving rmFH had lower plasma C3 and FH levels than they had 24 hours after the first dose. However, despite low levels of FH and C3 at 11 days (24 hours post-final dose), glomerular C3 and C3d staining remains at a reduced level. Both rmFH and rhFH appear to be immunogenic, with evidence of IgG deposition in the glomeruli and anti-FH antibody formation at the 11-day timepoint.

6.5 Experiment Three: Immunogenicity

The presence in $CFH^{-/-}$ mice of murine FH-related proteins (39, 84, 86), with their extensive sequence similarities to key regions of mFH (Figure 1.9),

implies these animals might not develop a strong immune response to rmFH despite lacking their own mFH. Figure 6.11 and 6.17, however, suggest rmFH is in fact immunogenic in *CFH*^{-/-} mice. To discover whether immunogenicity in *CFH* knockout mice is an inherent property of murine FH or arises from the recombinant nature of the mFH used in current studies, a further experiment was performed (Dr Kevin Marchbank and Dr Yi Yang, Institute of Cellular Medicine, University of Newcastle). In this experiment, 20 µg of either serum-derived native mFH or rmFH was injected *i.p.* on days 0 and 28 into either WT or *CFH*^{-/-} mice, and the outcome compared to that in vehicle-alone controls. Serum samples were taken, and tested for the presence of anti-FH antibodies, every seven days with a terminal bleed at 41 days (Figure 6.19). Due to the availability of animals, WT mice were eight weeks of age, while *CFH*^{-/-} mice were 4.5 – 6 months old at the start of this experiment.

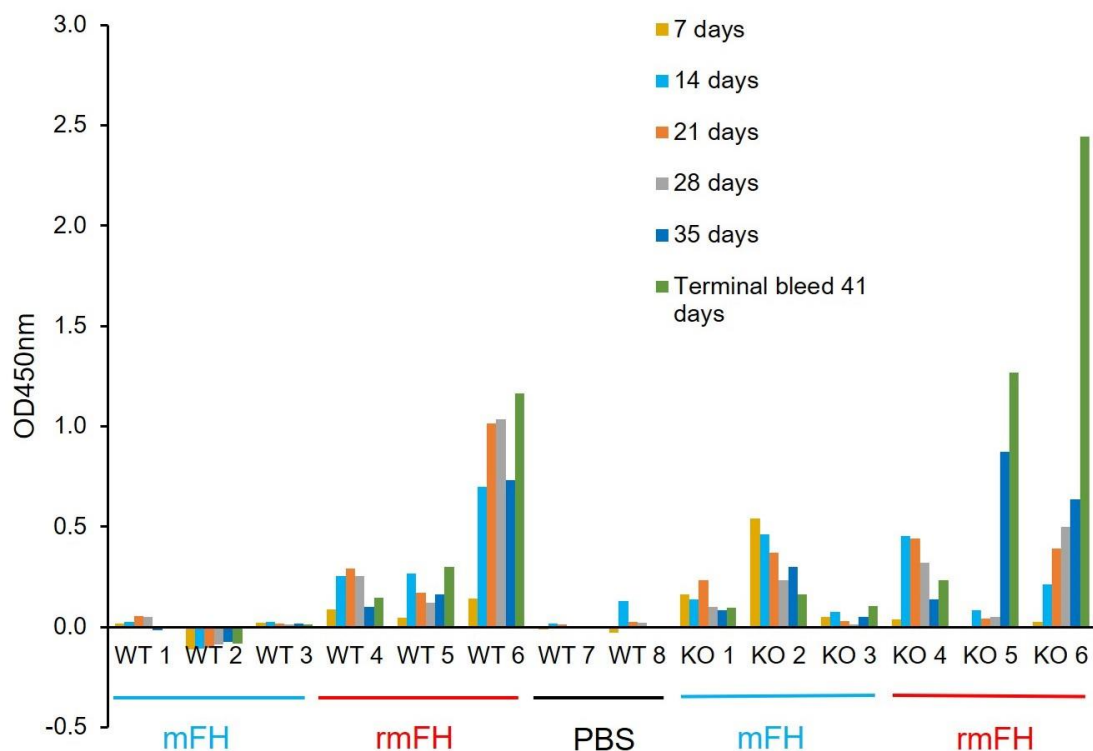


Figure 6.19 Presence of anti-FH antibodies after immunization with mFH, rmFH or PBS. WT: wild-type mice; KO: knockout *i.e.* *CFH*^{-/-} mice. OD 450 nm represents read-out of HRP-conjugated sheep anti-mouse IgG antibody (Jackson ImmunoResearch). mFH: mouse FH from serum; rmFH: recombinant murine FH. WT1 died at 35 days.

When WT mice received mouse serum-derived FH (mFH, WT 1,2,3 in Fig. 6.19), they do not mount a significant immune response against the FH protein. This is as expected, as these mice possess their own native mFH. However, when WT mice receive rmFH (rmFH, WT 4,5,6), they do develop anti-FH antibodies. In contrast, *CFH*^{-/-} mice injected with mouse serum-derived FH (mFH, KO 1,2,3) mount a smaller immune response relative to *CFH*^{-/-} mice (rmFH, KO 4,5,6) that receive rmFH.

Figure 6.20 shows the results of an ELISA to compare the immune-reactivity of the mouse terminal bleed serum samples generated with antigens of different origin. The serum of each mouse was tested against both native mFH and recombinant mFH. WT mice dosed with native mFH (WT 1,2,3) show only low reactivity to both native mFH and rmFH. WT mice dosed with rmFH (WT 4,5,6) make antibodies to rmFH (as seen in Figure 6.19) but show no reactivity to native mFH. This implies that antigenic differences between native mFH and rmFH lead to the development of an anti-rmFH immune response WT mice. *CFH*^{-/-} mice that received native mFH (KO 1,2,3) show a smaller immune response to both mFH and rmFH, compared to *CFH*^{-/-} mice that received rmFH (KO 4,5,6), which showed a large response to, importantly, *both* rmFH *and* mFH. It appears that the administration of rmFH to a factor H-deficient mouse results in a loss of tolerance to native mFH.

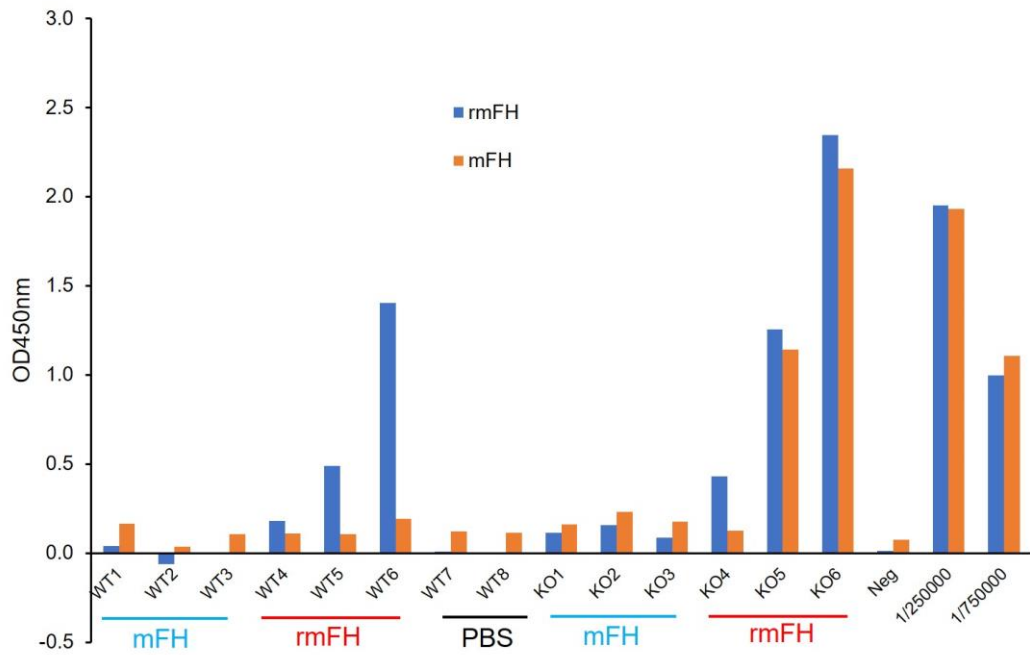


Figure 6.20 Presence of anti-FH antibodies in terminal bleed serum samples. WT: wild-type mice; KO: knockout ($CFH^{-/-}$) mice. OD 450 nM represents read-out of HRP conjugated sheep anti-mouse IgG antibody (Jackson ImmunoResearch). mFH: mouse FH from serum, rmFH: recombinant murine FH. WT1 died at 35 days. Neg: negative control, PBS alone. 1/250000 and 1/750000: positive control, mouse monoclonal antibody to mFH (Professor Claire Harris).

The experiment was repeated in a cohort of younger $CFH^{-/-}$ mice (eight weeks of age), to be comparable with original experiments. Figure 6.21 shows that, in agreement with Figure 6.19, $CFH^{-/-}$ mice that receive mFH (KO 2,3,4) developed a small number of anti-mFH antibodies, compared to $CFH^{-/-}$ mice that receive rmFH (KO 5,6,7), which show a large anti-FH antibody response.

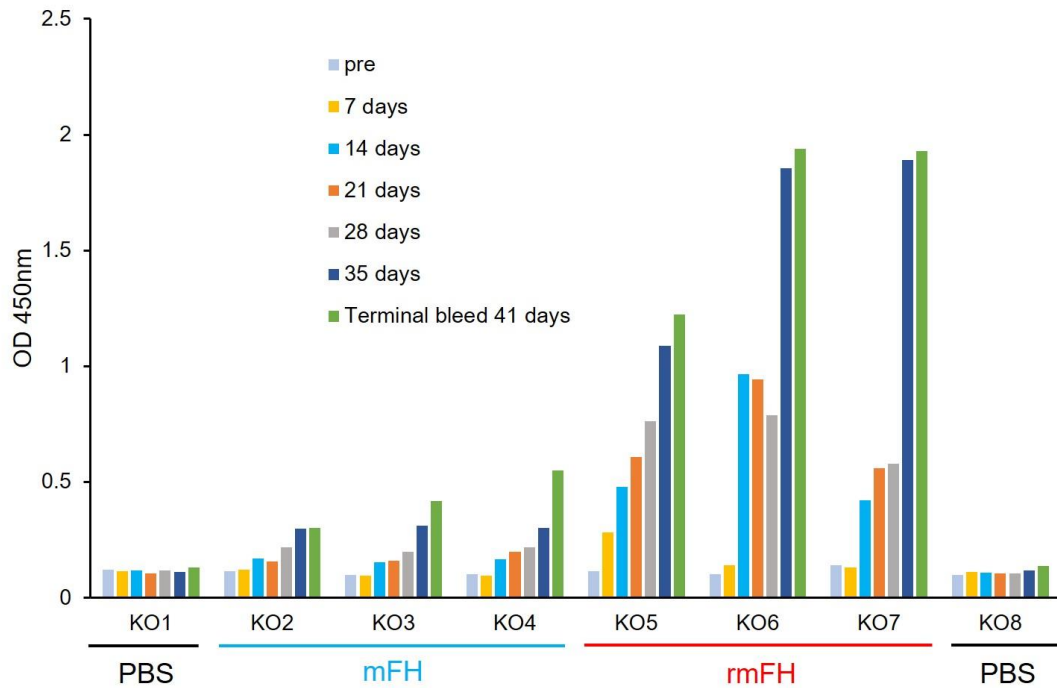


Figure 6.21 Presence of anti-FH antibodies after immunization with mFH, rmFH or PBS. KO: *CFH*^{-/-} mice. OD 450 nm represents read-out of HRP conjugated sheep anti-mouse IgG antibody (Jackson ImmunoResearch). mFH: mouse FH from serum, rmFH: mouse recombinant FH.

In conclusion, despite the presence of FH-related proteins in mouse plasma, rmFH is immunogenic and appears to be more so than native, serum-purified mFH, in both WT and *CFH*^{-/-} mice. A response to rmFH is evident in WT mice which does not cross-react significantly with native FH, suggesting antigenic epitopes exist on rmFH that are distinct to the native mFH in these mice.

6.6 Discussion

The aim of the *in vivo* experiments was to test the hypothesis that administration of recombinant murine (rm)FH, produced in yeast, can restore regulation of the alternative pathway (AP) of complement in *CFH*-knockout mouse models of C3 glomerulopathy. Based on previous results obtained from administration of serum-sourced (native) FH, it was anticipated that rmFH injections would restore regulation of C3 consumption in plasma, in conjunction with clearing C3 deposits from the glomerular basement

membrane. As presented in Figures 6.5 and 6.6, rmFH administration does indeed achieve these goals, although it fails to increase plasma C3 levels to the same extent as the equivalent dose of native FH purified from mouse serum (Figure 6.4). The relatively rapid *in vivo* clearance of rmFH prompted trials of a ten-day regimen of daily dosing. Daily administration of both rmFH and similarly produced human (h)FH murine led to the production of more anti-FH antibodies and greater deposition of IgG in the glomerulus than did dosages of the native protein (Figures 6.12 and 6.17). Thus an immune response is launched against the recombinant protein. An important observation was that *CFH*^{-/-} mice treated with rmFH develop antibodies against not only rmFH but also native mFH (Figure 6.20). Thus, as a proof of concept for the potential use of recombinant FH as a therapeutic protein to treat C3G, these results generate several points for discussion.

6.6.1 Experimental design

A homozygous FH-deficient (*CFH*^{-/-}) murine model was used in this work since it is the best currently available animal model of the rare kidney disease, C3G. Pickering *et al.* (119) showed that in two-month old mice, linear subendothelial electron-dense deposits were seen in the capillary wall, while C3 and C9 deposits were detected by immunofluorescence. This group further reported a lack of IgG staining in these deposits; and noted that renal function, assessed on the basis of serum albumin, creatinine and urine protein loss, was normal at this age. For the current studies, *CFH*^{-/-} mice aged 7-9 weeks were used.

The availability of *CFH*^{-/-} mice and FH protein defined the design of Experiments One and Two. Only twelve mice were available for each experiment. In Experiment One, just two mice received mFH and two were dosed with PBS (vehicle) Four mice received 0.5 mg rmFH and four got 1 mg rmFH, respectively. In Experiment Two, just two mice received rhFH, compared to five receiving 0.5 mg rmFH and five receiving PBS. Ideally an equal number of mice would have received each protein/vehicle to make comparisons easier. At the time of experimental planning, it was decided to give either 0.5 mg (or 1 mg) of both native and recombinant murine FH protein

to *CFH*^{-/-} mice (Experiment One). Given that recombinant proteins lack N-glycans and have a smaller MWt, it might have been better to adjust the doses to make them equimolar. In any case, the availability of rhFH limited its application to 0.4 mg per dose in Experiment Two, making for a problematic comparison with the 0.5 mg rmFH dose.

In Experiment One, a single, 0.5-mg *i.p.* dose of native mFH resulted, after 24 hours, in higher plasma FH and C3 levels than 0.5 mg of recombinant mFH protein. In Experiment Two, despite daily dosing of rmFH, FH and C3 levels had fallen by the five-day timepoint. It would have been advantageous to have included serum-purified mFH, for comparison, in this experiment, but there was insufficient murine serum available to act as a source of the additional mFH required. Assessment of murine albuminuria, urea/creatinine and histological studies would also have been worthwhile.

6.6.2 Rapid plasma-clearance of recombinant murine factor H

Exogenous human FH has an estimated half-life of six days in human FH deficiency (229) and was detectable in circulation four days after injection in *CFH*^{-/-} mice (139). Following a single *i.p.* injection of rmFH into *CFH*^{-/-} mice, plasma FH levels were elevated at six hours but dropped back by 24 hours. Conversely, but not unexpectedly (138), FH remained elevated in the plasma of *CFH*^{-/-} mice 24 hours after injection of native mFH. Rapid clearance of rmFH has previously been noted for a version of full-length hFH with partially humanized N-glycans produced in moss (228), whilst a short half-life was also observed for “mini” hFH (missing modules 6-17) produced in CHO cells (230) and a mouse recombinant protein composed of mouse CR2 (1-4) and mFH (1-5) (231). All were virtually undetectable after six hours. Full-length FH is too large for glomerular filtration (232), but the presence of GlcNAc residues, recognised by Man/GlcNAc receptors (e.g. on macrophages), could render rmFH subject to rapid clearance (233, 234). The lack of mammalian glycosylation could also predispose rFH to rapid clearance by natural antibody/innate immune recognition (235).

Native hFH carries eight disialylated N-glycans (20) and human FH has polyanion-binding sites in domains 7 and 20 (24). The lack of negatively charged N-glycans on our recombinant FH is a plausible explanation for an increased avidity for extracellular matrix (ECM) and hence its more rapid disappearance from plasma. This could simply be an electrostatic effect, or another theory, predicated on the postulated existence of *intramolecular* contacts between FH-borne sialic acids and sialic acid-binding sites on FH, proposes that when binding to recombinant (non-glycosylated) FH, GBM (ECM) sialic acids would not have to compete with covalently attached sialic acids (24). The lack of a mFH-fluorescent labelled antibody or protein precluded an experiment to test this explanation directly. It was previously shown that CHO cell-produced fluorescently tagged recombinant mFH was detectable in the GBM shortly after injection into *CFH*^{-/-} mice (236). Notably, Paixão-Cavalcante *et al.* (138), after injection of 1 mg fluorescent-tagged native (*i.e.* glycosylated) mFH to *CFH*^{-/-} mice, did not detect mFH along the GBM. They had previously found reactivity of glomeruli from unmanipulated *CFH*^{-/-} mice with a polyclonal anti-FH antibody, and had interpreted this reactivity as a consequence of cross-reactivity with FH related proteins. Nichols *et al.* (230), injecting ‘mini’ hFH into *CFH*^{-/-} mice, also detected FH in the glomerulus (detection was performed using the OX-24 anti-human FH primary antibody, which does not detect murine FH and therefore could not be used in our study).

6.6.3 Development of anti-FH antibodies to recombinant murine FH

Elevated plasma levels of rFH were unsustainable even with ten sequential daily injections. A similar observation was reported (139) following multiple daily injections of native human FH. By day five, in the current study, levels of recombinant FH were at 50% of WT and this appeared insufficient to arrest a declining trend in C3 levels, presumably because replenishment did not keep pace with consumption. The further deterioration of FH (and C3) levels between five and eleven days is likely connected to the emergence of anti-FH antibodies. *CFH*^{-/-} mice might be expected to have some immune tolerance to

mFH due to their possession of mFH-related proteins (mFHR) (39, 86). Indeed, in a separate 41-day experiment (with FH injected at days 0 and 28), we did not detect a higher titre of anti-mFH antibodies in *CFH*^{-/-} mice than in wild-type mice, when both groups were treated with native (mouse serum-derived) mFH. This experiment also showed that rmFH is more immunogenic than native mFH in wild-type mice, and that rmFH was more immunogenic in *CFH*^{-/-} mice than in wild-type mice. The anti-FH antibodies induced in wild-type mice by rmFH selectively recognized rmFH, suggesting that epitopes might include the GlcNAc residues or the “frayed ends” of the clipped backbone. It would be interesting to test the reactivity of the antibodies to shorter fragments of the recombinant protein e.g. the N- and C- terminus plus the ‘clipped’ section, to assess specificity of the antibodies.

6.6.4 Recombinant murine FH reduces glomerular C3 staining in *CFH*^{-/-} mice at doses that incompletely restore plasma C3 levels

Tubulo-interstitial staining for C3 is present in healthy WT mice. In unmanipulated *CFH*^{-/-} mice it is absent; complete resolution of GBM C3 staining is seen in *CFH*^{-/-} kidneys transplanted into WT hosts (72) with a concomitant appearance of a normal tubulo-interstitial C3 staining pattern. When WT kidneys were transplanted into *CFH*^{-/-} animals, tubulo-interstitial staining was lost in the WT transplanted kidney. Therefore C3 staining within the interstitium is dependent on the presence of intact circulating C3.

Administration of recombinant versions of mFH and hFH temporarily raised plasma levels of intact C3. Dose for dose, however, they resulted in smaller and shorter-lived elevations of intact C3 than did native mFH. No C3-staining in mesangial or interstitial tubular regions was detected in rrmFH-treated mice consistent with sub-wild-type levels of C3 in plasma. These observations may be explained as secondary consequences of the more rapid clearance of recombinant FH, given that plasma levels of intact C3 reflected plasma levels of FH.

In previous multiple daily-dose experiments using native hFH, staining with polyclonal anti-C3 antibody declined after five days but reappeared after ten days (139). This breakdown in the propensity of daily native hFH injections to control C3 fragment deposition between five and ten days was linked to anti-hFH antibodies that appeared during this time. In the current study treatment with rmFH likewise significantly reduced glomerular anti-C3 staining, compared to *CFH*^{-/-} mice receiving PBS. But, unlike in the previous study, this reduction persisted beyond the tenth daily injection of recombinant mFH, despite low FH and C3 levels in plasma.

Plasma levels of FH do not necessarily reflect how much FH is present in the GBM where FH can bind many partners including GAGs, sialic acids (see above) and, if present, C3 fragments. Thus recombinant FH could become trapped, with a long residency time. When FH is continuously supplied by intra-peritoneal injection, and notwithstanding the increasing titre of circulating anti-FH antibodies, a steady-state quantity of functional FH could be established in the GBM that is adequate for both preventing any further local generation and deposition of nascent C3b and proteolytic processing of existing C3b. Indeed, in both the previous and current studies, staining for the C3d fragment of C3b was also reduced, compared to controls, after ten days of recombinant mFH treatment.

A serendipitous scenario, in which circulating recombinant FH and C3 levels are low while recombinant FH residency in the GBM is high, could be optimal from the narrow perspective of resolving C3 fragment deposition in glomeruli. It is reminiscent of the *CFH*^{-/-}/*CRRY*^{-/-} double-knockout mouse in which a mild phenotype (compare to *CFH*^{-/-}) was attributed to plasma C3 depletion (236). It is the inverse of the scenario underlying the severe phenotype associated with homozygosity for the mFH mutant W1206R (237) (equivalent to disease-related mutant W1183R in hFH). In these animals fluid-phase AP regulation and hence C3 levels are maintained but C3b amplification on surfaces is unregulated because W1183R has only a poor affinity for surfaces. The benefit observed in our study might be temporary because in the longer term, higher

titres could develop of anti-FH antibodies or antibodies arising to the GBM-bound form of FH, and antibody-antigen complexes could accumulate in the kidney.

An obvious way to reduce the immunogenicity of our recombinant murine FH would be to use a strain of *P. pastoris* that decorates glycoproteins with the desired N-glycans (238) to yield FH that has a native conformation and immune tolerance. Serendipitously however, we show that a good, although not necessarily clinically desirable, means of resolving C3 fragment deposition in glomeruli is to allow C3 consumption in fluid phase to be largely unchecked while targeting regulation to the GB.

7: Conclusions

Factor H is considered the most important regulator of the AP of complement. Mutations or deficiencies of FH lead to the development of diseases with significant morbidity and mortality. The *CFH*^{-/-} murine model is analogous to human C3GN, with low plasma C3 levels and deposition of C3 in the glomerulus. Administration of serum-purified mFH (138) or hFH (139) were found to raise plasma C3 levels and reduce C3 staining in the glomerulus, although prolonged administration of hFH resulted in the development of anti-FH antibodies. It was postulated whether recombinant FH replacement could be a potential treatment for patients with C3GN.

A major hurdle to such an approach would be the very large quantities of protein needed. An alternative would be to augment the FH already present in patients. Therefore the aim of this project was as follows:

1. Generate, biophysically and functionally characterise full-length recombinant murine factor H (rmFH) in yeast
2. Administer rmFH to prevent development of C3GN in *CFH*^{-/-} mice
3. Administer rmFH to ameliorate renal injury due to C3GN in *CFH*^{-/-} mice
4. Produce full-length mutant variants of rhFH and characterise their function in conjunction with PspCN

Given the previous success in our laboratory with the microbial production of functional recombinant human FH (23), it was hoped that recombinant murine factor H, codon-optimised for production in the yeast *Pichia pastoris* and using the same expression vector as had been successful for rhFH, would be straightforward. However this proved not to be the case. The lack of evidence for even low-yield production of rmFH led us to try a new codon-optimised gene. This appeared more promising in small-scale tests, but even production *via* large-scale fermentation yielded small amounts of purified rmFH despite multiple attempts at optimisation of fermentation conditions. The uncommonly high number (40) of disulphides in FH suggested that boosting of levels of the chaperone protein, protein disulphide isomerase (PDI), might be needed. The

cloning of a PDI gene into a new base *Pichia* strain resulted in a marked improvement in the production of rmFH with yields enhanced by two orders of magnitude. Unfortunately, a proportion of the recovered rmFH protein showed evidence of proteolytic 'clipping', which could not be fully ameliorated despite the use of alternative protease inhibitors during the fermentation stage. Nor could clipped protein be resolved from unclipped protein (in non-reducing conditions) despite extensive purification attempts. A small amount of enriched non-clipped rmFH (rmFH-CHA) was achieved by purification using a ceramic hydroxyapatite column, but it was not practical to purify large amounts of rmFH in this way due to the large amount of protein lost during this purification step. Over 100 mg of purified (clipped) rmFH was achieved in the end, a significant achievement given the initially poor yields. This use of PDI paves the way for the production of other disulfide-rich multiple-domain recombinant proteins in significant quantities.

The rmFH was biophysically and functionally characterised and compared to rmFH-CHA (non-clipped) and serum-purified (native) mFH. Recombinant mFH binds to murine C3b, the key activation-specific fragment of C3, and accelerates the decay of the murine C3 convertase (C3bBb) as effectively as native mFH. No difference was found in the functional *in vitro* assays between samples containing clipped rmFH and rmFH-CHA (non-clipped). Interestingly, neither rmFH nor native mFH were able to decay the human C3 convertase, although rhFH did decay mC3bBb. This could be relevant to interpretation of the previous study using hFH to "treat" *CFH*^{-/-} mice. There is currently no published literature that has fully characterised murine FH in this way.

N-terminal sequencing identified that the clipped area of rmFH occurred in CCP 15 in an eight-amino acid residue insertion that lies within what is likely a disordered region that has been called the "hypervariable loop". It is possible that, within the context of the native glycoproteins, this area may be shielded by N-glycans (yeast high-mannose glycans are removed during purification of recombinant material).

Due to the significant issues and delays in achieving successful production of rmFH, some of the planned murine experiments were not possible. Prevention of disease experiments would have included administration of rmFH twice weekly for two months to eight-week old *CFH*^{-/-} mice. A further experiment would have examined amelioration of renal injury in *CFH*^{-/-} mice that were six months old. Instead, experiments were focussed on proof of concept of the functionality of rmFH administered to *CFH*^{-/-} mice. The first experiment compared single doses of rmFH and native, serum-purified, mFH. The results showed: i) high levels of mFH and intact C3 persist in plasma 24 hours after dosing *CFH*^{-/-} mice with native FH; ii) dosing with rmFH temporarily raised plasma mFH levels but rmFH was cleared faster than native mFH; iii) injected rmFH controlled fluid-phase C3 consumption less effectively than did native mFH, presumably due to faster clearance; iv) the protective effects of rmFH, in terms of preventing C3 fragment deposition along the GBM, persisted after serum levels of the administered recombinant protein had fallen to 50% of wild-type levels.

The second experiment compared daily injections for ten days, of rmFH and rhFH. Ideally plasma-purified mFH should have been included as a comparison here, particularly to assess for anti-FH antibodies, but the amount of protein required for this (10 mg total for two mice at 0.5 mg a day) was not available. This experiment showed that after five days of daily dosing, *CFH*^{-/-} mice receiving rmFH had lower plasma C3 and FH levels than they had 24 hours after the first dose. However, despite low levels of FH and C3 at 11 days (24 hours post-final dose), glomerular C3 and C3d staining remained at a reduced level. Both rmFH and rhFH appeared to be immunogenic, with evidence of IgG deposition in the glomeruli and anti-FH antibody formation at the 11-day timepoint.

Given the development of anti-hFH antibodies after administration of plasma-purified hFH for ten days (139), it was not unexpected that anti-hFH antibodies occurred in this experiment. However it was postulated that due to the presence of murine FHRs and their similarity to mFH, anti-mFH antibodies may

not develop. Comparing mFH with mFHRs however (Figure 1.9), shows that the N-terminal CCPs 1-4 of mFH are not represented in the mFHRs, and therefore could be an immunogenic focus. Despite this however, rmFH appears consistently more immunogenic than plasma-purified mFH, in both WT and *CFH*^{-/-} mice as seen in Experiment 3 (Figure 6.20), suggesting that epitopes might include the GlcNAc residues or the “frayed ends” of the clipped backbone. Notably, none of the more recent published studies on either moss-produced rFH (228) or ‘mini’ FH (230, 239) given to *CFH*^{-/-} mice have performed extended dosing experiments, nor looked for the presence of anti-FH antibodies. All, however, showed a short half-life.

Recombinant mFH was deglycosylated during the purification process (leaving residual GlcNAcs), to aid purification (non-deglycosylated protein appears as a ‘smear’ on SDS PAGE). Moreover, the *Pichia* sugars would be inherently immunogenic to mice due to differing glycosylation patterns between yeasts and mammals (yeast have high-mannose sugars, mammals have complex N-glycans with terminal sialic acids). N-glycans are rarely integral to the functionality of proteins (240), but they have been widely observed to enhance thermal stability, provide protection from proteolysis, improve solubility and inhibit aggregation of proteins (235). Recombinant mFH was prone to aggregation at cool temperatures and concentrations > 1.5 mg/ml, and the removal of the residual GlcNAcs to reduce immunogenicity could have exacerbated this issue.

Given the inherent issues with non-native glycosylation of recombinant proteins, there has been development of humanized yeast strains to produce complex terminally sialylated glycoproteins (238). This is a not insignificant process, however, involving multiple gene insertions, and the yeast strains are not widely available. Hence it was not explored in the current project.

Since starting this project, Ding *et al* (83) developed a humanised FH murine line (crossed with *CFH*^{-/-} mice) expressing the H402 SNP that predisposes to DDD (123). These mice have glomerular changes similar to *CFH*^{-/-} mice, but crucially have a low level of circulating human FH and so may abrogate the

issue of anti-hFH antibodies if dosed with human FH as this will not be perceived as a foreign protein (compared to *CFH*^{-/-} mice who lack any FH at all).

When this project was conceived over ten years ago there was no consensus treatment for patients with C3GN, and this remains the case. This is partly due to the rarity of the disease meaning large clinical trials are more challenging, but also due to the underlying heterogeneous nature of the condition, with many different underlying complement abnormalities. It is increasingly recognised however that targeting of the C3 convertase or its substrate C3 could be a successful treatment rationale for C3GN. This is a developing field of complement therapeutics with several promising contenders such as Cp40, a peptidic C3 inhibitor which has been awarded orphan designation from the European Medicines Agency for the treatment of C3G (241, 242). Pegcetacoplan (Apellis Pharmaceuticals, www.clinicaltrials.gov) is a C3 inhibitor undergoing Phase III clinical trials in patients with C3G.

A further emerging field is the development of small molecule inhibitors against complement proteins, in particular FB and FD. Novartis Pharmaceuticals is currently recruiting into APPEAR-C3G, a comparison of Iptacopan (FB inhibitor) with placebo in patients with C3G. Danicopan, a FD inhibitor (Alexion Pharmaceuticals, www.clinicaltrials.gov) underwent a Phase II trial to determine the effect of the drug on C3 levels in C3G patients. The advantage of both these molecules is their oral administration. The RENEW study aims to also evaluate BCX9930 (a FD inhibitor, Biocryst Pharmaceuticals, www.clinicaltrials.gov) in patients with C3G.

Avacopan is a C5a receptor inhibitor which through the ACCOLADE trial (ChemoCentryx, www.clinicaltrials.gov) has shown promising results in improving the renal function of patients with C3G.

There have yet to be any clinical trials evaluating the use of full length FH in C3G or aHUS. Gemini Therapeutics has developed a recombinant full length human FH (GEM103) (www.gemini therapeutics.com) for the treatment of dry

age-related macular degeneration. This was shown to have similar in vitro functional activity (243) as native FH although further clinical trial results are awaited at this point. The feasibility of full-length FH in treating patients with C3G or aHUS therefore remains unknown. However, the successful production of aHUS-associated mutants of rhFH and their characterisation, with the bacterial virulence protein PspCN, has helped lay the groundwork for a therapy based on 'rescuing' some of the deleterious effects of mutations.

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Appendices

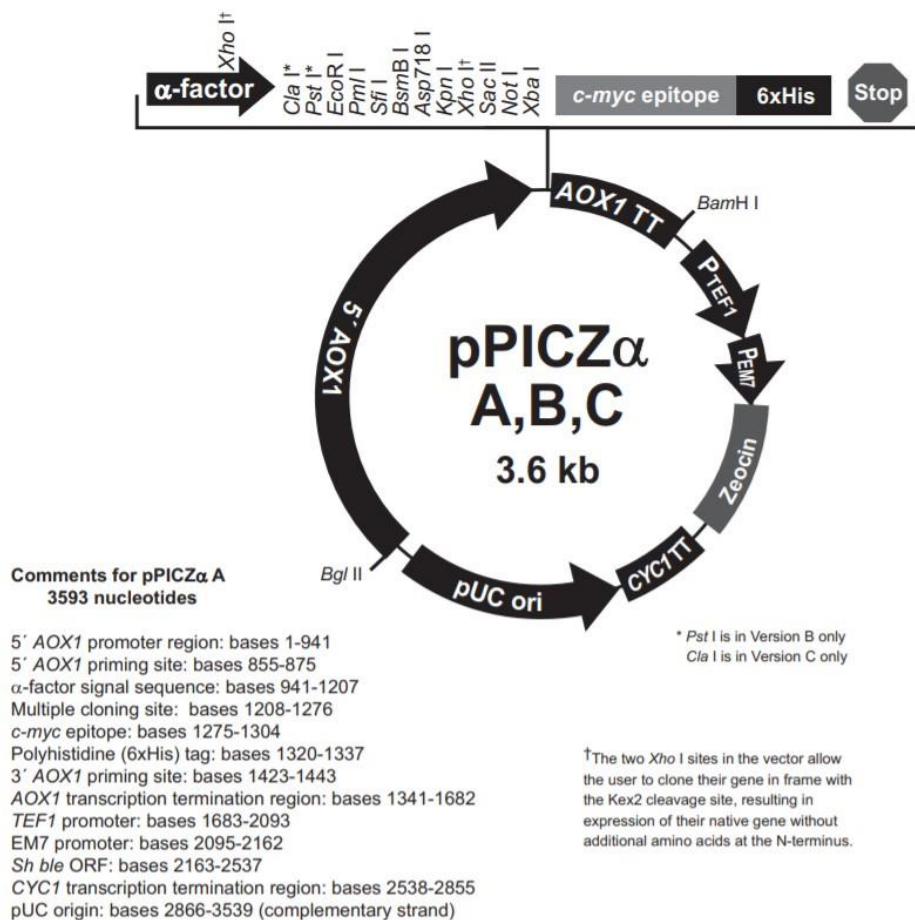
Appendix A: Media and buffers

MEDIA	COMPOSITION (w/v unless stated otherwise)
BMG (Buffered minimal glycerol)	100 mM potassium phosphate pH 6 1.34 % YNB (Yeast Nitrogen Base) 4×10^{-5} % biotin 1 % glycerol
BMGY (Buffered glycerol complex)	100 mM potassium phosphate pH 6 1.34 % YNB 4×10^{-5} % biotin 1 % glycerol 1 % yeast extract 2 % peptone
BMMY (Buffered methanol complex)	100 mM potassium phosphate pH 6 1.34 % YNB 4×10^{-5} % biotin 0.5 % v/v methanol 1 % yeast extract 2 % peptone
LB (Luria-Bertani)	0.5 % yeast extract 1 % tryptone 1 % sodium chloride +/- 1.5 % agar

Low-salt LB	0.5 % yeast extract 1 % tryptone 0.5 % sodium chloride +/- 1.5 % agar
MD (Minimal dextrose)	1.34 % YNB 4×10^{-5} % biotin 2 % dextrose +/- 1.5 % agar
YPD (Yeast extract, peptone, dextrose)	1 % yeast extract 2 % peptone 2 % dextrose (D-glucose) +/- 2 % agar
YPDS (Yeast extract, peptone, dextrose, sorbitol)	1 % yeast extract 2 % peptone 2 % dextrose (D-glucose) 1 M Sorbitol +/- 2 % agar
YPG (Yeast extract, peptone, glycerol)	1 % yeast extract 2 % peptone 1 % glycerol
TAE buffer 50x in distilled H ₂ O	Tris-acetate (2 M) EDTA (100 mM)

Appendix B: *Pichia* vectors

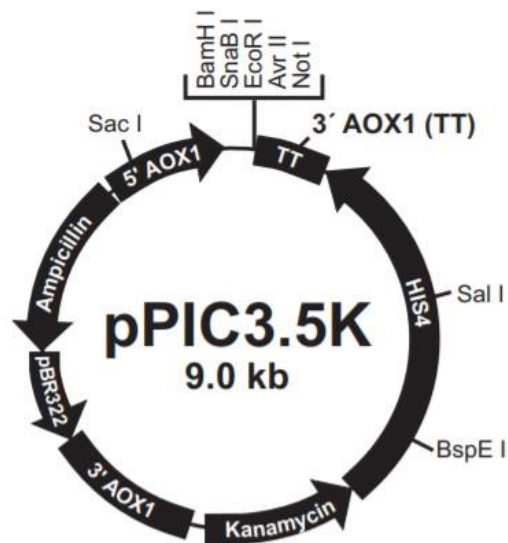
1: pPICZαB



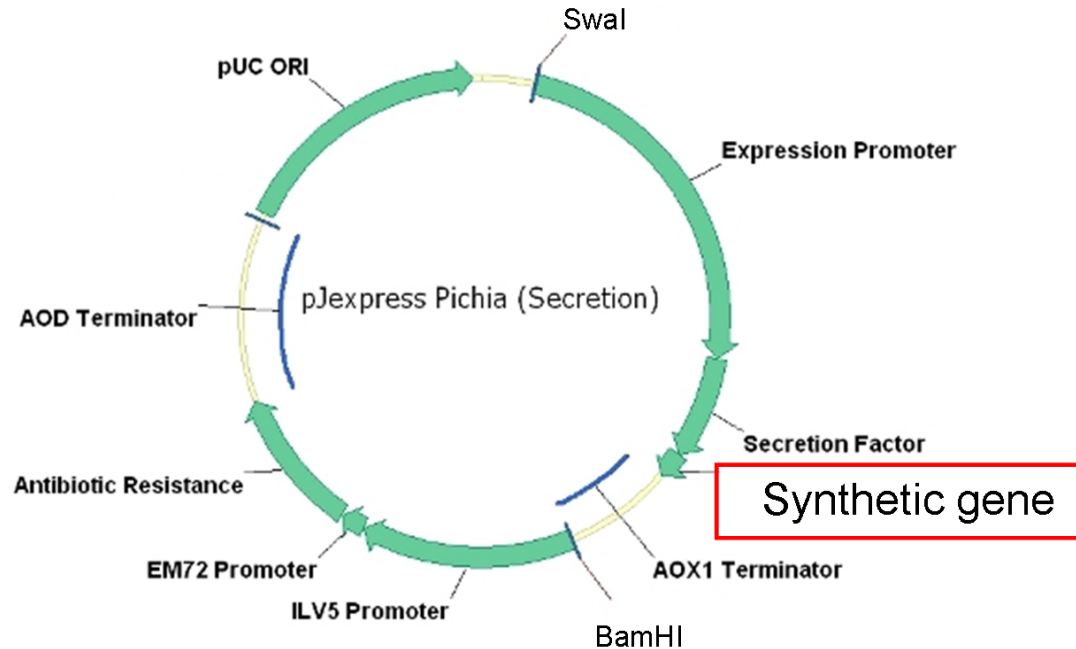
2: pPIC3.5K

Comments for pPIC3K: 9004 nucleotides

5' AOX1 promoter fragment: bases 1-937
 5' AOX1 primer site: bases 855-875
 Multiple Cloning Site: bases 938-968
 3' AOX1 primer site: bases 1055-1075
 3' AOX1 transcription
 termination (TT): bases 981-1314
 HIS4 ORF: bases 4242-1708
 Kanamycin resistance gene: bases 5458-4656
 3' AOX1 fragment: bases 5850-6607
 pBR322 origin: bases 7689-7016
 Ampicillin resistance gene: bases 8694-7834



3: pJexpress912



Appendix C: Recombinant murine factor H sequencing primers

	Forward primer 5' – 3'	Reverse primer 5' – 3'
1	CAAGCTACTTACAAGTGTAGACCAGGT	GGATGGTTGAGAAGCAACCCACTT
2	TCCGGTTTCAAGATCGAAGGTCAC	CAAAATCTCAACACATCTTGGCTT
3	GGTTGGATTCCAGTTCCAAGA	GTTTGGTCTCAAGGACTCTTCGTA
4	ATCAAGACTTGTTCCGCTTCC	GGAAGTCTCTCTGTTCAAAGCGTA
5	AAGTTGGTTGTTTCCCAAGA	ACATTGAACGGAGTCTGGACC
6	TCCGTTGAGTTCATCTGTGAGGAA	ACACTTTGGCAACTGAGTCCA
7	TCCGAAGAGATGGTTTGTAAGGAC	GTGCTCAATAGTTGGTGGCTG
8	TGTGAGGGTGGTAAGTGGTCTGAT	CTTGGACTIONTCTGATGATAGCGTT
9	ACTGGTAAGTGTGGTCCTCCA	TTGGTACTCAACGGAGGACAA

Appendix D: Optimisation of Western blot

Western blot	Primary Antibody dilution used (supplier)	Secondary Antibody dilution used (supplier)
a)	Rabbit antibody to mouse FH 1:200 (Santa Cruz)	Goat antibody to rabbit IgG 1:1000 (Cell Signalling)
b)	Goat antibody to human FH 1:200 (Calbiochem)	Rabbit antibody to goat IgG 1:50,000 (Sigma)
c)	Sheep antibody to human FH 1:200 (Abcam)	Rabbit antibody to sheep IgG 1:20,000 (Abcam)

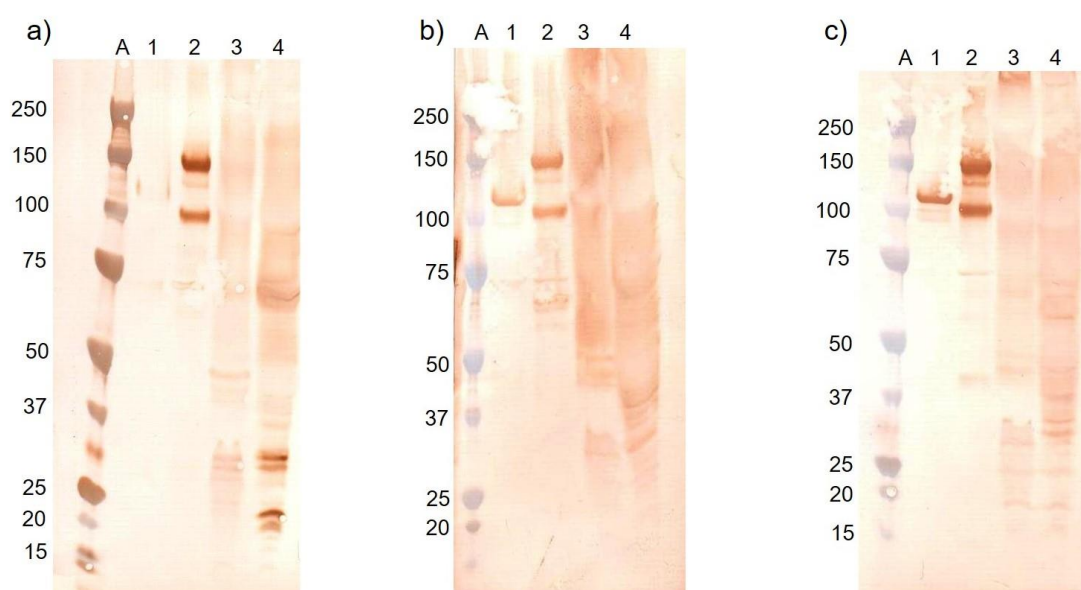


Figure 7.1 Comparison of antibodies in detection of recombinant murine factor H. 1: rmFH 0.4 μ M, non-reduced. 2: rmFH 0.4 μ M, reduced. 3: KM71H supernatant (untransformed), non-reduced 4: KM71H supernatant (untransformed), reduced. A: protein MWt markers. All wells were loaded with 10 μ l of solution.

Appendix E: ELISA protocol – rhFH production in KM71H and PDI-KM71

This ELISA was performed by Dr Eliza Makou (Figure 3.26).

	1	2	3	4	5	6	7	8	9	10	11	12
A	FH 5 µg/ml	FH 2.5 µg/ml	...	...								0 µg/ml
B	As in A1	As in A2	...	...								0 µg/ml
C	X	Y	Z	etc								
D												
E												
F												
G												
H												

All wells were coated with 8 µg/ml PspCN.

Wells A1-A12 and B1-B12 contain Complement Technologies-sourced human plasma-derived FH as control, series of dilutions starting from 5 µg/ml.

Wells C1-12, D1-12 contain diluted supernatant, in duplicates

Reagents

Plate:

Flat-bottomed, FALCON, 96 wells

Capture antibody coating buffer:

NaHCO₃ 0.1 M, pH 9.5, 250 ml

2.1 g of sodium bicarbonate pH adjusted with NaOH

Wash buffer:

1 litre: 1 x PBS plus Tween 20 (0.5 ml)

Blocking buffer:

100 ml 1 x PBS plus 5 % w/v milk powder (5 g milk powder for 100 ml)

Dilution buffer:

1 x PBS plus Tween 20 plus 5 % w/v milk powder (5 g milk powder for 100 ml)

OPD (o-Phenylenediamine dihydrochloride) (Sigma):

Immediately before use, dissolve 10 mg OPD powder in 18 ml H₂O and add 2 ml of stable peroxide buffer to the dissolved OPD.

Sulphuric acid:

200 ml of a 2.5 M solution: slowly add 27 ml of stock acid to 173 ml H₂O.

His-tag SUMO PspCN (stock: 8 mg/ml):

For 8 µg/ml 1:1000 dilution to cover plate (96 wells, 100 µl each = 9.6 ml), so made 15 ml: 15 µl stock PspCN (gift from Dr Andrew Herbert) in 15 ml capture buffer.

Add 100 µl to all wells using multistep pipette.

Complement Technologie-sourced FH in dilution buffer:

Stock FH is supplied at 1 mg/ml but 5 µg/ml starting dilution was needed (2.5 µl in 500 µl).

Put 100 µl dilution buffer in all wells columns **2-12**, rows A and B

Put 200 µl 5 µg/ml in A1 and B1, mix six times, then take 100 µl into column 2 and mix six times, and so on, until column 11. Once the 100 µl is added to column 11 and mixed, take 100 µl out and discard. Column 12 contains only 100 µl buffer.

Factor H small-scale test expressions:

Make a 1:10 dilution of the supernatant in dilution buffer: e.g. 60 µl supernatant and 540 µl dilution buffer. Add 100 µl samples to wells.

Detection antibody (primary): OX-24 Biotin *Lifespan Biosciences* LS-C62878.

1:800 dilution of the stock and 10 mL are required. Hence dissolve 12.5 µl stock in 10 ml dilution buffer, or 18.75 µl stock in 15 ml dilution buffer.

Use 100 µl for ALL wells.

Streptavidin-peroxidase polymer ultrasensitive (secondary) Sigma S-2438.

1:250 dilution of stock needed. Hence for 10 ml, 40 µl of stock in 10 ml dilution buffer.

Use 100 µl in each well.

PROCEDURE:

Incubations on Belly Dancer orbital shaker (Sigma)

1. Add 100 µl of PspCN (8 µg/ml) to each well using a multistep pipette. Put lid on the plate.
2. Incubate 600 mins and transfer to the cold room, with no shaking, overnight.
3. Add 200 µl blocking buffer to all wells using a multistep pipette. Incubate for one hour at room temperature.
4. Using a multistep pipette remove buffer from all wells. Remove excess fluid.
5. Add 200 µl wash buffer to each well. Remove buffer with pipette and excess fluid. Repeat twice.
6. Add FH controls to wells with coordinates, A1-11 and B1-11, as mentioned in previous section.
7. Add 100 µl dilutions of the supernatants from any test expressions.
8. Incubate for one hour, at room temperature.
9. Repeat steps 4 and 5, above.
10. Add 100 µl OX-24 dilution to each well.
11. Incubate for one hour, at room temperature.
12. Repeat steps 4 and 5 above.
13. Add 100 µl streptavidin peroxidase solution to each well.
14. Incubate for one hour, at room temperature. Ten minutes before the end, make the OPD.
15. Repeat steps 4 and 5, above.
16. Add 100 µl OPD to each well. Leave 10-30 mins. Read OD at 450 nm if needed. Observe the colour change.
17. Add 100 µl sulphuric acid to stop the reaction in the order you added the OPD.
18. Read OD at 490 nm.

Values of FH concentrations correspond to the 1:10 dilution so multiply observed value by ten to get actual value.

Appendix F: Recombinant sequences

Recombinant murine factor H DNA sequence (DNA 2.0)

GAAGATTGTAAAGGTCCTCCGCCTAGAGAGAACTCGGAAATCTTGTTCAGGTTCTGCTG
TGAGCAACTCTATCCAGAGGGTACGCAAGCTACCTACAAATGCCGTCCAGGTTATAGAA
CACTTGGAACCATCGTCAAGGTTTGTAAAAACGGAATGGGTTGCCTCAAATCCCTCC
AGAATTTGTCGTAAAAAGCCATGTGGTCATCCGGGTGATACACCTTTTCGGTTCCTTCAG
ATTGGCAGTCGGTTCCTCAATTTGAATTTGGTGCAAAAGTCGTGTACACTTGTGATGATG
GTTATCAGCTGTTGGGTGAAATTGACTACAGGGAATGTGGTGCTGATGGTTGGATCAAT
GACATTCCATTGTGTGAAGTCGTGAAGTGTCTACCAGTTACTGAATTGGAAAATGGCAG
AATTGTTTCCGGAGCAGCCGAAACCGATCAGGAGTACTACTTCGGTCAAGTTGTTTCGTT
TCGAGTGTAATTCTGGCTTCAAGATTGAGGGACATAAGGAGATCCACTGTTCCGAAAAT
GGTTTGTGGAGCAATGAGAAACCAAGATGTGTGGAGATTTTGTGTACTCCTCCTAGAGT
CGAGAATGGCGACGGTATAAACGTAAAACCTGTTTACAAGGAAAACGAAAGATACCATT
ACAAGTGCAAGCACGGTTATGTGCCTAAGGAAAGGGGTGATGCTGTCTGCACAGGCTC
AGGTTGGAGCAGCCAGCCTTTTTGCGAGGAAAAGAGATGTAGCCCACCATATATCTTAA
ACGGAATCTACACCCACATAGGATCATCCACCGTTCGGACGATGAAATCCGTTATGAA
TGTAACACGGCTTCTACCCTGTTACAGGTTCAACTGTCAGTAAGTGTACGCCAACTGG
TTGGATTCTGTTCCAAGATGTACACTTAAACCTTGTGAATTTCCACAATTCAAGTATGG
CAGGTTGTACTATGAAGAGTCATTACGTCCGAATTTCCAGTCTCGATCGGCAACAAGT
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CTTAAGGTTCAATGTTACAATGGTTACTCTCTTCAAAACGGTCAAGATACTATGACATGT
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CCATCACATGTTTGCAAAATGGATGGAGCCCACAGCCCTCTTGCAATTAAGTCTGTGAT
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TCCCTTGTTCCCAGCCACCTACAATAGAACACGGTTCCATAAACCTGCCAAGATCGTCT
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TGAGGACATCGAGTTTGGTTGTAAGTATGGATATTACAAAGCTAGAGACTCACCACCAT
TCAGGACAAAAGTGATAAACGGTACTATCAACTACCCAACTTGT GTTTAG

Recombinant murine factor H amino acid sequence (UniProt P06909)

EDCKGPPPRENSEILSGSWSEQLYPEGTQATYKCRPGYRTLGTIVKVCKNGKWWASNPSRI
CRKKPCGHPGDTPFGSFR LAVGSQFEFGAKVVYTCDDGYQLLGEIDYRECGADGWINDIPL
CEVVKCLPVTLENGRIVSGAAETDQEYFGQVVRFECSGFKIEGHKEIHCSENGLWSNE
KPRCVELCTPPRVENG DGINVKPVYKENERYHYKCKHGYVPKERGDAVCTGSGWSSQPF
CEEKRCSPPYILNGIYTPHRIIHRSDDEIRYECNYGFYPVTGSTVSKCTPTGWIPVPRCTLKP
CEFPQFKYGRLYYEESLRPNFPV SIGNKYSYKCDNGFSPPSGYSWDYLRCTAQGWEPEVP
CVRKCVFHYVENGD SAYWEKVYVQGQSLKVQCYNGYSLQNGQDTMTCTENGWSPPPKCI
RIKTCSASDIHIDNGFLSESSSIYALNRETSYRCKQGYVTNTGEISGSITCLQNGWSPQPSCI
KSCDMPVFENSITKNRTRWFKLNDKLDYECLVGFENEYKHTKGSITCTYYGWS DTPSCYER
ECSVPTLDRKLVVSPRKEKYRVGD LLEFSCHSGHRVGPDSVQCYHFGWSPGFPTCKGQV
ASCAPPLEILNGEINGAKKVEYSHGEVVKYDCKPRFLLKGP NKIQCV DGNWTTLPVCIEEER
TCGDIPELEHGS AKCSVPPYHHGDSVEFICEENFTMIGHGSVSCISGKWTQLPKCVATDQL
EKCRVLKSTGIEAIKPKLTEFTHNSTMDYKCRDKQEYERSICINGKWDPEPNCTSKTSCPPP
PQIPNTQVIETTVKYLDGEKLSVLCQDNYLTQDSEEMVCKDGRWQSLPRCIEKIPCSQPPTI
EHGSINLPRSSEERRDSIESSSHEHGTTFSYVCD DGFRIPEENRITCYMGKWSTPPRCVGL
PCGPPPSIPLGTVSLELESYQHGE EVTYHCSTGFGIDGPAFIICEGGKWSDPPKCIKTDCDV
LPTVKNAIIRGKSKSYRTGEQVTFRCQSPYQMNGSDTVTCVNSRWIGQP VCKDNSCVDP
PHVPNATIVTRTKNKYLHGDRVRYECNKPLELFGQVEVMCENGIWTEKPKCRDSTGKCGP
PPPIDNGDITSLSLPVYEPLSSVEYQCQKYLLKGKKTITCRNGKWSEPTCLHACVIPENIM
ESHNIILKWRHTEKIYSHSGEDIEFGCKYGYKARDSPPFRTK CINGTINYPTCV

***Pichia pastoris* Protein Disulphide Isomerase (PDI) DNA sequence (GeneArt)**

GGATCCAAAACGATGCAGTTCAACTGGGACATCAAGACTGTTGCTTCCATCTTGTCCGC
 TTTGACTTTGGCTCAAGCTTCTGACCAAGAGGCTATTGCTCCAGAAGATTCCCACGTTG
 TTAAGTTGACTGAGGCTACTTTTCGAGTCTTCATCACTTCCAACCCACACGTTTTGGCTG
 AGTTTTTCGCTCCATGGTGTGGTCACTGTAAGAAGTTGGGTCCAGAATTGGTTTTCCGCT
 GCTGAGATTTTGAAGGACAACGAGCAGGTTAAGATCGCTCAGATCGACTGTACTGAAGA
 GAAAGAGTTGTGTCAGGGTTACGAGATCAAGGGTTACCCAACCTTTGAAGGTTTTCCACG
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 AGAGGTAAGATCAACTTCGTTGGTTTGGACGCTGTTAAGTTCGGTAAGCACGCTAAAAA
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 GAAGTTCCGAGTTCCACAGGACCAAGAGTTGACTAACAAGGACGTTACTGAGTTGATC
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 TCTGAAGCTGATGCTCACGATGAGTTGTAAGCGGCCGC

***Pichia pastoris* PDI amino acid sequence (UniProt B3VSN1)**

MQFNWDIKTV	ASILSALTTLA	QASDQEAIAP	EDSHVVKLTE	ATFESFITSN
PHVLAEFFAP	WCGHCKKLGP	ELVSAAEILK	DNEQVKIAQI	DCTEEKELCQ
GYEIKGYPTL	KVFHGEVEVP	SDYQGQRQSQ	SIVSYMLKQS	LPPVSEINAT
KDLDDTIAEA	KEPVIVQVLP	EDASNLESNT	TFYGVAGTLR	EKFTFVSTKS
TDYAKKYTSD	STPAYLLVRP	GEEPSVYSGE	ELDETHLVHW	IDIESKPLFG
DIDGSTFKSY	AEANIPLAYY	FYENEEQRAA	AADI IKPFAK	EQRGKINFVG
LDAVKFGKHA	KNLNMDEEKL	PLFVIHDLVS	NKKFGVPQDQ	ELTNKDVTEL
IEKFIAGEAE	PIVKSEPIPE	IQEEKVFKLV	GKAHDEVVFD	ESKDVLVKYY
APWCGHCKRM	APAYEELATL	YANDEDASSK	VVIAKLDHTL	NDVDNVDIQG
YPTLILYPAG	DKSNPQLYDG	SRDLES LAEF	VKERGTHKVD	ALALRPVEEE
KEAEAEAESE	ADAHDEL-			

Recombinant human factor H amino acid sequence and mutants (UniProt P08603)

I62 Y402 D936 (I62-FH)

AAEDCNELPPRRNTEILTGSWSDQTYPEGTQAIYKCRPGYRSLGN¹IMVCRKGEW
VALNPLRKCQKRPCGHPGDTPFGTFTLTGGNVFEYGVKAVYTCNEGYQLLGEINY
RECDTDGWTNDIPICEVVKCLPVTAPENGKIVSSAMEPDREYHFGQAVRFVCNSG
YKIEGDEEMHCSDDGFWSKEKPKCWEISCKSPDVINGSPISQKIIYKENERFQYKCN
MGYEYSERGDAVCTESGWRPLPSCEEKSCDNPYIPNGDYSPLRIKHRTGDEITYQ
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FISESQYTYALKEKAKYQCKLGYVTADGETSGSITCGKDGWSAQPTCIKSCDIPVF
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ELPKIDVHLVPDRKKDQYKVGEVLKFSCKPGFTIVGPNSVQCYHFGLSPDLPICKE
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VWTQLPQCVAIDKLKKCKSSNLIILEEHLKNKKEFDHNSNIRYRCRGKEGWIHTVCI
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EEITCKDGRWQSIPLCVEKIPCSQPPQIEHGTINSSRSSQESYAHGTKLSYTCEGG
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CFEGFGIDGPAIAKCLGEKWSHPPSCIKTDCLSLPSFENAIPMGEEKDVYKAGEQV
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GERVRYQCRSPYEMFGDEEVMCLNGNWTEPPQCKDSTGKCGPPPPIDNGDITSF
PLSVYAPASSVEYQCQNLYQLEGNKRITCRNGQWSEPPKCLHPCVISREIMENYNI
ALRWTAQKLYSRTGESVEFVCKRGYRLSSRSHTLRTTCWDGKLEYPTCAKR**

V62 Y402 E936 (V62-FH)

AAEDCNELPPRRNTEILTGSWSDQTYPEGTQAIYKCRPGYRSLGNVIMVCRKGEW
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GRKFVQGKSIDVACHPGYALPKAQTTVTCMENGWSPTPRCIRVKTCSSIDIENG
FISESQYTYALKEKAKYQCKLGYVTADGETSGSITCGKDGWSAQPTCIKSCDIPVF
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VWTQLPQCVAIDKLKKCKSSNLIILEEHLKNKKEFDHNSNIRYRCRGKEGWIHTVCI
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YTCATYYKMDGASNVT CINSRWTGRPTCRDTSCVNPPTVQNAYIVSRQMSKYPS
GERVRYQCRSPYEMFGDEEVMCLNGNWTEPPQCKDSTGKCGPPPPIDNGDITSF
PLSVYAPASSVEYQCQNLYQLEG NKRITCRNGQWSEPPKCLHPCVISREIMENYNI
ALRWTAQKLYSRTGESVEFVCKRGYRLSSRSHTLR TTCWDGKLEYPTCAKR**

I62 Y402 D936 (I62-FH base); R53H (R53H-FH)

AAEDCNELPPRRNTEILTGSWSDQTYPEGTQAIYKCPGYRSLGNIMVCRKGEW
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RECDTDGWTNDIPICEVVKCLPVTAPENGKIVSSAMEPDREYHFGQAVRFVCNSG
YKIEGDEEMHCSDDGFWSKEKPKCWEISCKSPDVINGSPISQKIIYKENERFQYKCN
MGYEYSERGDAVCTESGWRPLPSCEEKSCDNPYIPNGDYSPLRIKHRTGDEITYQ
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VGKYYSYYCDEHFETPSGSYWDHIHCTQDGWSPAUPCLRKCYPYLENGYNQNY
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FISESQYTYALKEKAKYQCKLGYVTADGETSGSITCGKDGWSAQPTCIKSCDIPVF
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ELPKIDVHLVPDRKKDQYKVG EVLKFSCKPGFTIVGPNSVQCYHFGLSPDLPICKE
QVQSCGPPPELLNGNVKEKTKEEYGHSEVVEYYCNPRFLMKGP NKIQCV DGEWT
TLPVCIVEESTCGDIPELEHGWAQLSSPPYYYGDSVEFNCSESFTMIGHRSITCIHG
VWTQLPQCVAIDKLKKCKSSNLIILEEHLKNKKEFDHNSNIRYRCRGKEGWIHTVCI
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EEITCKDGRWQSIPLCWEKIPCSQPPQIEHGTINSSRSSQESYAHG TKLSYTCEGG
FRISEENETTCYMGKWSSPPQCEGLPCKSPPEISHGVVAHMSDSYQYGE EVTYK
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GERVRYQCRSPYEMFGDEEVMCLNGNWTEPPQCKDSTGKCGPPPPIDNGDITSF
PLSVYAPASSVEYQCQNLYQLEG NKRITCRNGQWSEPPKCLHPCVISREIMENYNI
ALRWTAQKLYSRTGESVEFVCKRGYRLSSRSHTLR TTCWDGKLEYPTCAKR

I62 Y402 D936 (I62-FH base); D1119G (D1119G-FH)

AAEDCNELPPRRNTEILTGSWSDQTYPEGTQAIYKCRPGYRSLGN||IMVCRKGEW
VALNPLRKCQKRPCGHPGDTFPGTFTLTGGNVFEYGVKAVYTCNEGYQLLGEINY
RECDTDGWTNDIPICEVVKCLPVTAPENGKIVSSAMEPDREYHFGQAVRFVCNSG
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MGYEYSERGDAVCTESGWRPLPSCEEKSCDNPYIPNGDYSPLRIKHRTGDEITYQ
CRNGFYPATRGNTAKCTSTGWIPAPRCTLKPCDYPDIKHGGLYHENMRRPYFPVA
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GRKFVQGKSIDVACHPGYALPKAQTTVTCMENGWSPTPRCIRVKTCSSIDIENG
FISESQYTYALKEKAKYQCKLGYVTADGETSGSITCGKDGWSAQPTCIKSCDIPVF
MNARTKNDFTWFKLNDTLDYEC HDGYESNTGSTTGSIVCGYNGWSDLPICYEREC
ELPKIDVHLVPDRKKDQYKVGEVLKFSCKPGFTIVGPNSVQCYHFGLSPDLPICKE
QVQSCGPPPELLNGNVKEKTKEEYGHSEVVEYYCNPRFLMKGPNKIQCVDGEWT
TLPVCIVEESTCGDIPELEHGWAQLSSPPYYYGDSVEFNCSESFTMIGHRSITCIHG
VWTQLPQCVAIDKLKKCKSSNLIILEEHLKNKKEFDHNSNIRYRCRGKEGWHTVCI
NGRWDPEVNCSMAQIQLCPPPPQIPNSHNMTTTTLN YRDGEKVS VLCQENYLIQEG
EEITCKDGRWQSIPLCWEKIPCSQPPQIEHGTINSSRSSQESYAHGTKLSYTCEGG
FRISEENETTCYMGKWSSPPQCEGLPCKSPDISHGVVAHMSDSYQYGEEVTYK
CFEGFGIDGPAIAKCLGEKWSHPPSCIKTDCLSLPSFENAIPMGEKKDVYKAGEQV
TYTCATYYKMDGASNVTCINSRWTGRPTCRDTSVNPPTVQNAYIVSRQMSKYPS
GERVRYQCRSPYEMFGDEEVMCLNGNWTEPPQCKDSTGKCGPPPPIDNGGITSF
PLSVYAPASSVEYQCQNLYQLEG NKRITCRNGQWSEPPKCLHPCVISREIMENYNI
ALRWTAQKLYSRTGESVEFVCKRGYRLSSRSHTLR TTCWDGKLEYPTCAKR

I62 Y402 D936 (I62-FH base); S1191L V1197A (LA-FH)

AAEDCNELPPRRNTEILTGSWSDQTYPEGTQAIYKCRPGYRSLGN||IMVCRKGEW
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RECDTDGWTNDIPICEVVKCLPVTAPENGKIVSSAMEPDREYHFGQAVRFVCNSG
YKIEGDEEMHCSDDGFWWSKEKPKCWEISCKSPDVINGSPISQKIIYKENERFQYKCN
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FISESQYTYALKEKAKYQCKLGYVTADGETSGSITCGKDGWSAQPTCIKSCDIPVF
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VWTQLPQCVAIDKLKKCKSSNLIILEEHLKNKKEFDHNSNIRYRCRGKEGWHTVCI
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FRISEENETTCYMGKWSSPPQCEGLPCKSPDISHGVVAHMSDSYQYGEEVTYK
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GERVRYQCRSPYEMFGDEEVMCLNGNWTEPPQCKDSTGKCGPPPPIDNGDITSF
PLSVYAPASSVEYQCQNLYQLEG NKRITCRNGQWSEPPKCLHPCVISREIMENYNI
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Appendix G: recombinant murine factor H binding to human C3b

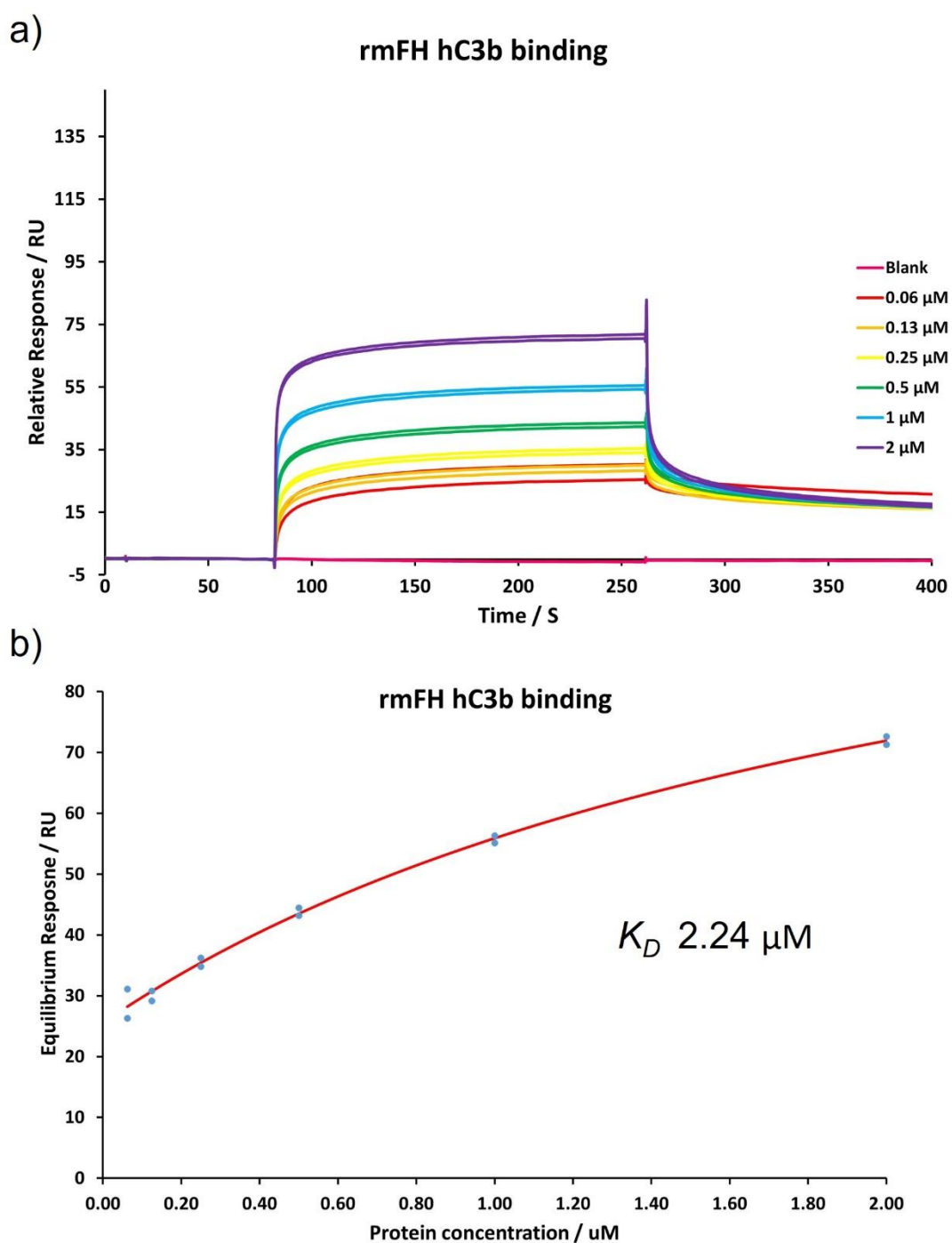


Figure 7.2 Binding of recombinant murine factor H to human C3b. a) Background-subtracted sensorgram with concentrations as per legend b) Plot of response versus concentration.